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PRINCIPAL INVESTIGATOR: Dr. Robert Humphreys
Dr. Minzhen Xu
Dr. Eric von Hofe
Dr. Xueqing Lu
Dr. Gilda Hillman

CONTRACTING ORGANIZATION: Antigen Express Inc
Worchester MA 01606

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Dr. Robert Humphreys; Dr. Minzhen Xu; Dr. Eric von Hofe; Dr. Xueqing Lu
Dr. Gilda Hillman

E-Mail: admin@antigenexpress.com

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14. ABSTRACT
Antigen Express has generated potent human Ii-RNAi constructs for use in prostate cancer therapy. We identified active Ii-RNAi sequences, selected the most active promoter (CMV) and demonstrated good suppression of Ii. The constructs also were used to enhance a gp120 DNA vaccine model, confirming that Ii suppression increases the potency of a tumor cell and DNA vaccine. We have shown that an anti-tumor immune response can be strongly induced by intratumoral induction of the MHC class I+/II+/Ii- phenotype and effectively and specifically protects mice from re-challenge by the same tumor cells. The frequency and dose of plasmid injections have been optimized as well as the dose of IL-2 to induce a potent immune response following generation of the MHC class I+/II+/Ii- phenotype. The duration of plasmid in cells in vitro and the in vivo have been evaluated and indicate that this immunotherapy should be safe. Finally, we have found that DU-145, PC-3, and LNcap prostate cell lines are heterogeneous for MHC class II and Ii expression and that PC-3 and LNcap cells are MHC class II-/Ii+. Overall, our results suggest that Ii suppression has the potential to be an elegant method for prostate cancer immunotherapy.

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INTRODUCTION

Antigen Express, Inc. has created a novel prostate cancer immunotherapy by converting tumor cells into antigen presenting cells (APC) *in vivo.* By presenting endogenous tumor antigens, such cells induce a potent T-helper cell-mediated immune response, which helps to activate CD8+ T cells and eradicate residual tumor and micrometastases. Tumor cells are converted into APC by inducing both MHC Class II expression and suppressing the immunoregulatory Ii protein. The Ii protein normally blocks the antigenic peptide binding site of MHC Class II molecules at synthesis in the endoplasmic reticulum and prevents MHC class II molecules from binding endogenous antigenic peptides that have been transported into that compartment. The therapeutic phenotype is therefore MHC Class I+/II+/Ii- tumor cells. By creating the MHC Class I+/II+/Ii- phenotype, tumor cells simultaneously present endogenous tumor antigens through both MHC Class I (normal pathway) and "unprotected" MHC class II molecules to activate both CD4+ and CD8+ T cells, generating a very potent tumor cell vaccine. Prior to this grant we had demonstrated the principle that *in situ* intratumor generation of MHC class II+/Ii- tumor cell phenotype was a potent therapeutic.

Our aims of this grant fall into three portions: **1)** Generate active Ii suppression constructs that can effectively induce MHC class II+/Ii- phenotype of tumor cells and optimize potent RNA interference constructs (Ii-RNAi) to inhibit Ii protein expression; **2)** Define the *in vivo* efficacy of MHC class II+/Ii-phenotype immunotherapy and the resulting elimination of metastatic tumor cells; along with determination of the frequency of immunization and definition of the optimal doses of plasmids that induce MHC class II+/Ii- phenotype; and **3)** Perform toxicology studies, including the bio-distribution of therapeutic reagents for a possible prostate cancer clinical trial. In the previous two annual reports, we have successfully generated the active reagents that effectively inhibit Ii expression in different tumor cell lines, including prostate cancer cell lines. The efficacy of MHC class II+/Ii- phenotype therapy has been clearly demonstrated by our collaborator, Dr. Hillman, and the doses of reagents and the frequency of immunization has also been determined. During the last year, we further optimized the application of reagents in prostate cancer cell lines and bio-distribution experiments have been carried out.

This final report describes the accomplishments related to the tasks we have set up in the grant. Some data is cited from the first and second annual reports and as well as data from the third year of this grant. In summary, we have successfully achieved our goals. Our results have justified clinical trials for prostate cancer immunotherapy as well as other human tumors using the methods and reagents identified here.

REPORT BODY

1. Generate active Ii suppression constructs that can effectively induce MHC class II+/Ii- phenotype of tumor cells. We have detailed the generation and optimization of potent RNA interference constructs (Ii-RNAi) to inhibit Ii protein expression (*from last two annual reports*).

a. Construction of Ii suppressing genetic constructs: Ii-RNAi. We previously constructed effective Ii-RGCs (Ii reverse gene constructs), which effectively inhibited Ii expression in many murine tumor cells. Likewise we previously constructed and validated active human Ii-RGCs. However, recent reports (in particular since the submission of the grant proposal) have shown

that RNAi technology is possibly a more effective and reliable method to silence expression of a given gene. Therefore, we constructed an Ii-RNAi to suppress human Ii expression in prostate cancer cells. However, since the DU145 human prostate cancer cell line is MHC class II-negative and Ii negative, we did the initial testing in Raji cells, a MHC class II+/Ii+ lymphoma cell line. Ten Ii-RNAi expression constructs were engineered in a pSuppressorAdeno plasmid (Imgenex, CA), following standard molecular biology techniques and instructions of the manufacturer. The Ii-RNAi sequences were designed according to either a computer algorithm of Imgenex or by inspection by our scientists. Raji cells were used for testing the Ii suppressing activity of these Ii-RNAi constructs. Raji cells were transfected *in vitro* with the Ii-RNAi constructs using gene gun delivery. Cells were then cultured for 18-24 hours and stained with anti-Ii and anti-HLA-DR antibodies and analyzed by flowcytometry for Ii and MHC class II expression. Three out of the ten Ii-RNAi constructs had significant activity in suppressing Ii protein expression. **Figure 1** shows that the three Ii-RNAi constructs significantly inhibited Ii expression in about 40-50% of cells (reflecting the transfection efficiency) without affecting expression of MHC class II molecules. The active constructs where used for optimization of the Ii suppression in the experiments planned in DU145 prostate cells, which is described below.

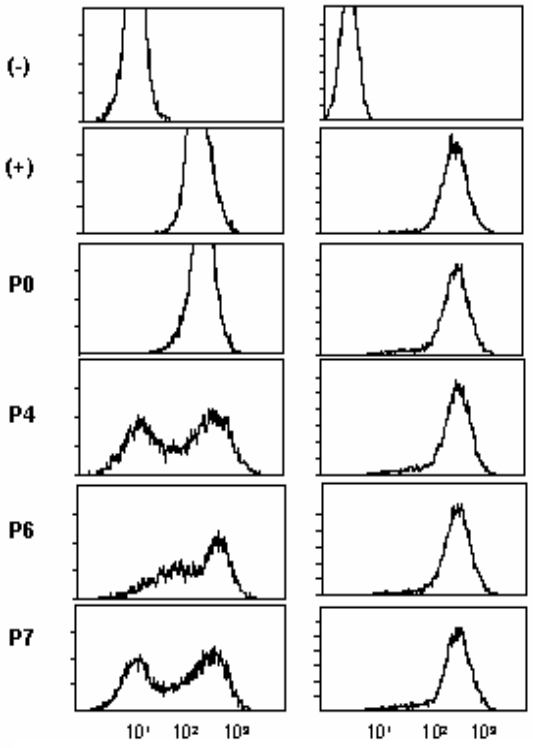

Figure 1. Ii inhibition in Raji cells. Cells were stained with anti-human Ii (left) and HLA-DR antibodies (right). P0 is empty plasmid control

b. Induction of MHC class II and Ii in DU145 human prostate tumor cells. DU145 is a well-studied, human prostate tumor cell line. It is a MHC class II-negative/Ii-negative tumor line. In order to effect Ii protein suppression in a MHC class II molecule-positive tumor, we had to first generate MHC class II+/Ii+ DU145 tumor cells. This effort mimics our requirement to work with MHC class II-negative, Ii protein-negative human prostate tumors within patients. Since MHC class II transacting factor (CIITA) is the master inducer of MHC class II molecules and Ii protein, we used a plasmid containing a gene encoding the CIITA protein to transfect the tumor cells. CIITA acted on regulatory elements preceding the respective structural genes to induce the expression of MHC class II and Ii protein. Transfection of the cells with CIITA was accomplished using the gene gun-mediated method to deliver the CIITA plasmid into the cells. Breifly, the gene was coated uniformly onto ultra-small gold beads, insuring quantitative delivery per cell. For each cartridge (one cartridge per shooting), 0.5 mg of 1 μm gold microparticles was used. The indicated amount (5-20 mg for 10-40 cartridges) of gold microparticles was suspended by sonication in 100 μl of 0.05 M spermidine. Total DNA at a concentration of 1 μg/ml in endotoxin-free water was added and sonicated; 100 μl of 1 M $CaCl_2$ was subsequently added drop wise. This gold-DNA mixture was washed 3 times using 250 μl of 100% ethanol and finally re-suspended with the indicated amount of 100% ethanol (1 ml for producing 17 coated 0.5-inch cartridges). The coated cartridges were stored at 4^0C with

desiccant prior to use. For transfecting DU145 cells, 10^6 cells in 20 ml medium was plated onto a tissue culture dish in about 1 cm diameter circles, which were subjected to gene gun shooting with one 0.5-inch cartridge using a helium pressure of 350 psi. After culturing, cells were stained with anti-MHC class II (FITC-conjugated anti-human HLA-DR) and anti-Ii monoclonal antibodies (anti-human CD174 plus FITC-conjugated anti-mouse IgG) and analyzed by flow cytometry to determine the percentage of MHC class II+/Ii+ cells. **Figure 2** shows the FACS profile of a representative experiment. One observes that more than 50% of DU145 cells have been induced to express MHC class II molecules and the Ii protein at 0.5 ug of CIITA/cartridge. The expression of MHC class II and Ii is CIITA dose-related.

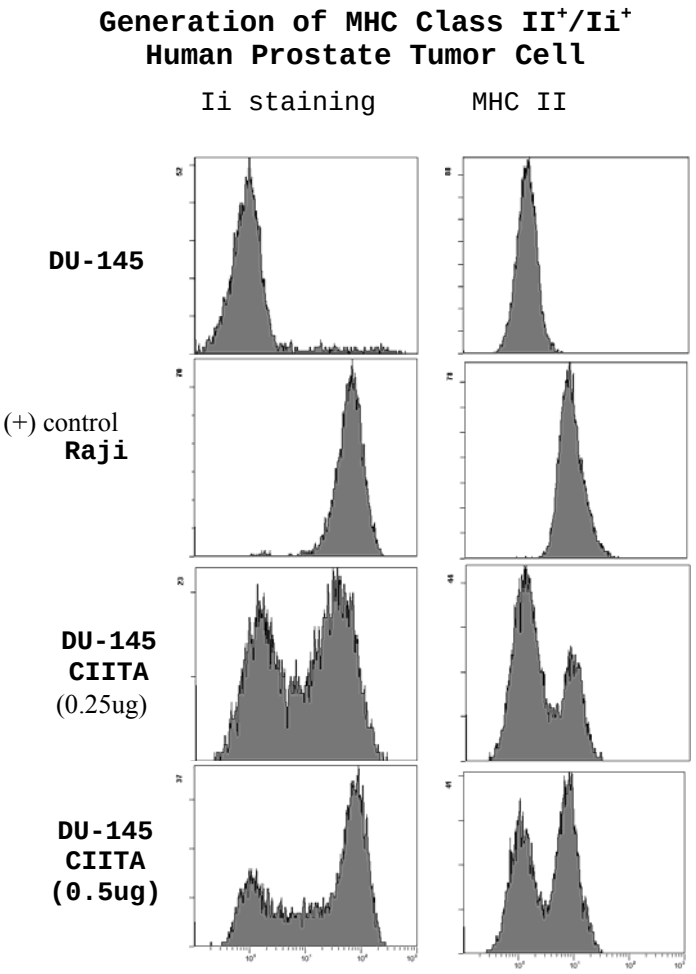

Figure 2. MHC class II and Ii protein induction by CIITA gene transfection Gene gun method was used to deliver the DNA.

c. Inhibition of murine invariant chain (Ii) in bone marrow-derived dendritic cells. In parallel to the above efforts to create effective siRNAs for use in human cells, we have pursued the design, synthesis and validation of a similar protocol for murine cells. The bulk of our efforts in Task 2 was to validate our reagents in murine models. We successfully demonstrated the inhibition of Ii in murine cells using synthetic and expressed siRNAs for the purpose of enhancing the presentation of MHC class II epitopes in antigen presenting cells. These experiments where first pursed in dendritic cells for two reasons. 1) They are already MHC class II-positive and Ii protein-positive, i.e., they do not need to be induced with CIITA, and 2) Suppression of Ii in DCs leads to additional ways to vaccinate against prostate cancer antigens, e.g., gene transduction for PSA or PSMA. In an effort to decrease murine Ii expression in antigen presenting cells, we synthesized siRNA molecules specific for murine Ii mRNA. Two siRNA molecules were introduced into murine dendritic cells and the J774 promyelocytic leukemia cell line, using chemically modified yeast glycan particles (YGP). The YGP particles are taken up by DCs in a receptor mediated process that results in efficient expression of plasmid DNA and delivery of synthetic siRNA. We have previously used a plasmid pIi RGCx3 that results in decreased expression of Ii protein when transfected into Ii positive cells. This plasmid was constructed using a portion of the Ii coding sequence inserted into an expression plasmid in the reverse orientation. Using an antibody specific for Ii and flow cytometry, the expression of Ii is detected on 66% of murine DCs as

shown in **Figure 3, C2**. Ii expression was not affected by empty YGP particles. Transfection of pIi RGCx3 resulted in a decrease in Ii expression, as shown in **Figure 3**.

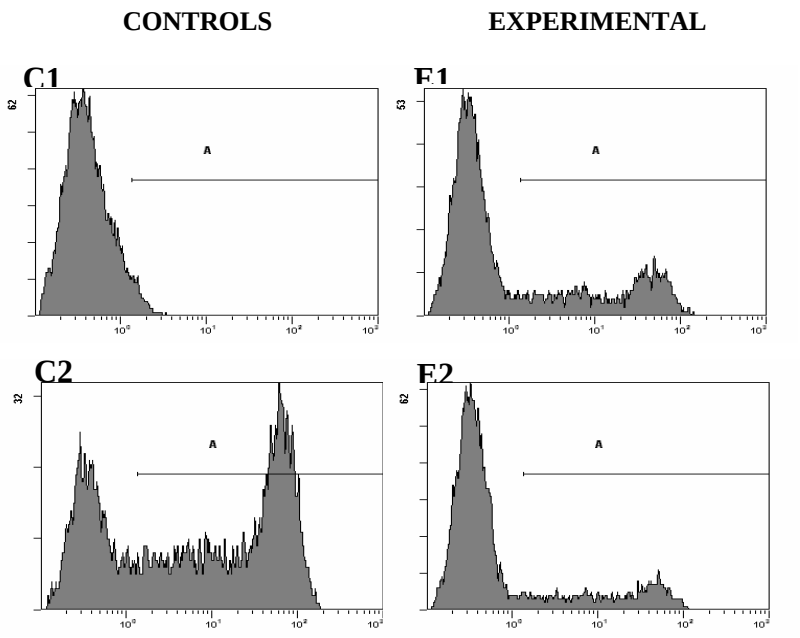

Figure 3 (C1) represents flow cytometric analysis of DC stained with only a secondary goat anti-mouse FITC antibody. (C2) DC fed with YGP were stained with a primary murine Ii-specific antibody and a secondary goat anti-mouse FITC-conjugated antibody. (E1) DC fed with YGP particles loaded with the Ii RGCx3 plasmid; stained with a primary murine Ii specific antibody and a secondary goat anti mouse FITC antibody. (E2) DC fed YGP loaded with synthetic siRNA specific for murine Ii were stained with a primary murine Ii-specific antibody and a secondary goat anti-mouse FITC-conjugated antibody.

For these experiments, bone marrow cells were extracted from the femurs of BALB/c mice. Cells were plated at $4x10^6$ cells in 100 mm dishes, in RPMI 1640-medium supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine (2 mM), 2-BME (50 ug/ml), and 10% fetal calf serum. The bone marrow cells were treated for 6 days with 20 ng/ml murine GM-CSF. For the remaining 4 days of incubation, the cells were given fresh medium with 10 ng/ml murine GM-CSF. At day 10, the immature dendritic were washed with PBS, trypsinized, plated in 6 well plates $5x10^5$ per 2 ml medium. The cells were fed chemically modified yeast cells, YGP particles. These particles bear a chemical group readily recognized by a receptor uniquely on DC, leading to the uptake of the particle into the DC and expression of DNA encapsulated in the particle. The YGP particles are taken up by DCs in a receptor-mediated process that results in efficient expression of plasmid DNA and delivery of synthetic siRNA. YGP particles where loaded with Ii RGCx3 plasmid DNA or siRNA. The Ii RGCx3 plasmid was constructed using a portion of the Ii coding sequence inserted into an expression plasmid in the reverse orientation. The RNAi plasmid resulted in expression of a biosynthetic siRNA which decreased expression of Ii protein, by an RNAi mechanism. Cells were harvested 48 hr after addition of the YGP particles. The cells were washed with PBS, formalin-fixed, quenched with glycine, and permeabilized with saponin in preparation for staining with Ii monoclonal antibodies (data not shown).

d. Optimization of combination use of human Ii-RNAi constructs.

Large bodies of the literature have shown that the combination use of the RNAi constructs that target different sites of an mRNA yields better inhibition of gene expression. Thus we have focused our efforts to optimize the combination use of Ii-RNAi constructs. The active Ii-RNAi constructs, P4, P6, and P7, target different sites of Ii mRNA. We performed experiments in which all combinations of P4, P6, and P7 were used, respectively, to inhibit the Ii expression in Raji cells. From **Figure 4**, one can see that all combinations of active human Ii-RNAi constructs showed better Ii suppression than single use of any of the three active Ii-RNAi constructs. Among these combinations, the P4/P7 combination gave the most profound Ii inhibition (the negative peak is much larger than other negative peaks of other combinations under same experiment condition). This result indicates that the combination use of P4 and P7 Ii-RNAi constructs is the best combination for Ii inhibition and this combination could potentially be our final formula for clinical trial.

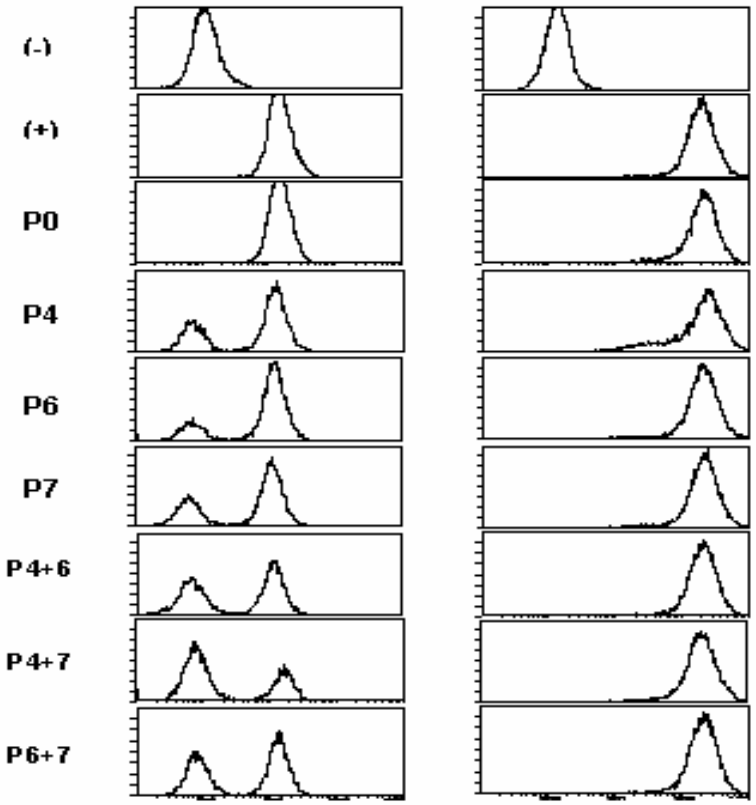

Figure 4. Combination use of Ii-RNAi in Raji cells. Raji cells were gene gun transfected with equal amount of Ii-RNAI constructs (For single use, 1 ug of DNA was used. For combination use, 0.5 ug of each plasmid was used). Cells were then incubated for another 48 hours and then stained for HLA-DR and Ii. P0 is empty plasmid control.

e. Test the influence of the promoter on the activity of Ii-RNAi constructs. In the early stage of constructing our Ii-RNAi constructs, we first cloned all ten Ii-RNAi fragments into a plasmid under the control of a U6 promoter. Transfection of Raji cells with these U6/Ii-RNAi constructs showed that all U6/RNAi constructs were inactive (data not shown). We changed the promoter and cloned all ten Ii-RNAi fragments into plasmids under the control of a CMV promoter. Transfection of Raji cells using the CMV promoter identified three active Ii-RNAi constructs (**Figure 1**). These results indicated that the promoter may play an important role in the activity of an Ii-RNAi construct in a given cell line. In an attempt to further confirm this idea, we chose an EF-1a promoter for further experiments. P4 and P7 Ii-RNAi fragments were cloned into a plasmid under the control of an EF-1a promoter. EF-1a is active in most mammalian cells. Transfection of an acute myeloid leukemia (AML) cell line, KG-1, with EF-1a/P4 and EF-1a/P7 constructs indicated that these two constructs were active in KG-1 cells while the transfection of

KG-1 cells with CMV/P4 and CMV/P7 is relatively inactive (**Figure 5**). Our result indicates that the activity of a promoter is important for the RNAi activity in a given cell line or a given type of cells, paving the way for using our human Ii-RNAi constructs in different tumor models.

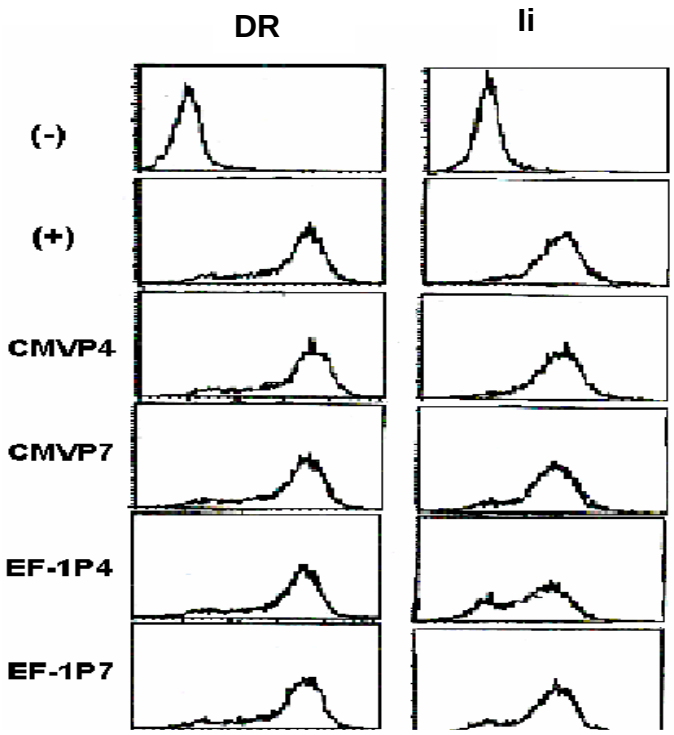

Figure 5. Influence of EF-1a promoter and CMV promoter in the activity of Ii-RNAi activity. KG-1 cells were gene gun transfected with CMV/P4, CMV/P7, EF-1a/P4, and EF-1a/P7, respectively. Cells were then stained with anti-HLA-DR and Ii antibodies and FACS analyzed.

f. Activity of human Ii-RNAi constructs in primary AML cells. In order to pursue a clinical trial with our Ii-RNAi constructs, the constructs must be active in primary tumor cells. We have used AML cells as samples to test this idea. This experiment was performed by our collaborator, Dr. Daopei Lu. Primary AML cells were collected from newly diagnosed AML patients. Cells were then frozen for further use. The AML cells were thawed and incubated for 24 hours before gene gun-mediated DNA transfection. Cells were transfected with EF-1a/P4 and EF-1a/P7 constructs, incubated for another 48 hours, collected and stained with anti-HLA-DR and anti-human Ii monoclonal antibodies and then FACS analyzed. The experiments indicate that the EF-1a/P4 and EF-1a/P7 Ii-RNAi constructs were active in primary AML cell samples (data not shown).

g. Define the enhancement of Ii suppression in DNA vaccine. We have also tested Ii suppression for the enhancement of the potency of a DNA vaccine. The rationale is the same as for Ii suppression to enhance the potency of tumor cell vaccine. When an APC acquires both DNA for an antigen and for Ii-RGC plasmid, the APC becomes an antigen+/class I+/class II+/Ii- phenotype. In that APC, antigen will be synthesized and processed as endogenous antigen and presented through both class I and class II pathways. Gp120 cDNA was used as our experimental model. GM-CSF DNA was used as a DNA adjuvant and a triple murine Ii-RGC was used as the Ii suppression reagent. All DNAs were mixed in a specific ratio (see Figure 6 legend) and then coated onto gold beads. A gene gun was then used for delivery of DNA. The potency of Ii suppression to enhance HIV gp120 DNA vaccine efficiency was tested in BALB/c mice. Mice were immunized with the gene for gp120 and for GM-CSF, with or without the Ii suppression construct. Two epitopes (p18, restricted by H-2D^d and H-2A^d, and p18-I10, restricted by H-2D^d) were used to measure the immune response to the gp120 antigen. In **Figure 6**, one sees that both p18- and p18-I10-specific ELISPOT assays demonstrated roughly 5 times the enhancement of IFN-γ secreting cells in the Ii-suppressed groups (groups D and E) compared to the Ii un-suppressed group (group C). The enhancement was related to the Ii suppression and not related

to the use of GM-CSF. The enhancement was more profound at the lower concentration of pBudCE4.1/Ii-RGC(x3) group (compare group D to group E). This phenomenon may reflect less promoter competition among gp120-, GM-CSF-, and Ii-RGC-containing plasmids. Similar reaction patterns to p18 and p18-I10 stimulation were observed, the p18 peptide gave a greater response in most reactions. This result is consistent with previous reports which show p18-I10 is restricted only by H-2D^d while the p18 peptide is restricted by both H-2D^d and H-2A^d. The p18 reaction reflects both CD4+ and CD8+ reactions and p18I-10 reaction reflects only CD8+ reaction. In order to determine whether Ii suppression induced a Th1 or Th2 response, IL-4 secretion was also examined in the ELISPOT assay. IL-4 was induced in all groups (Figure 6). Compared to IFN-γ production, IL-4 production was lower, indicating that the addition of the GM-CSF gene induced a Th1-biased immune response.

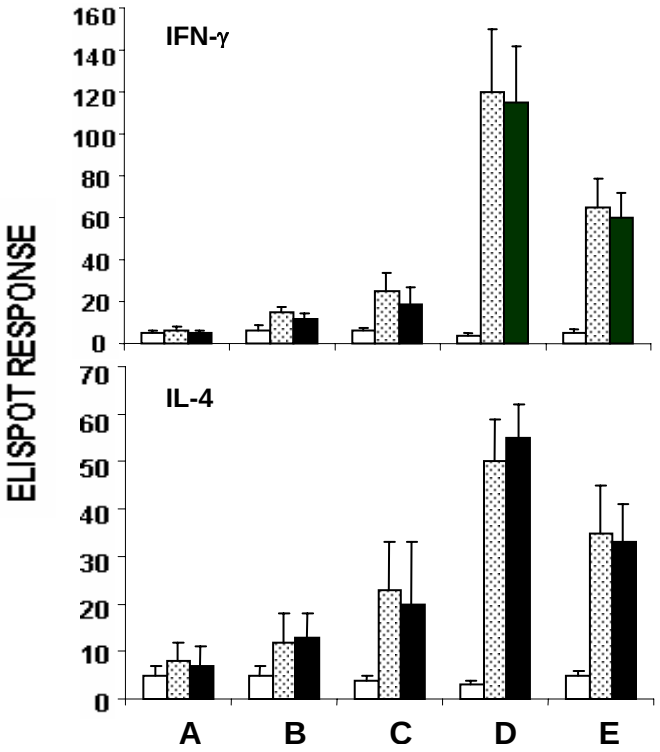

Figure 6. IFN-γ and IL-4 ELISPOT assays with splenocytes of mice immunized with gp120 with or without Ii suppression. All groups except A) (naïve mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and each of the following DNA plasmids, respectively: B) Empty pBudCE4.1 (1.35 μg); C) pNGVL1/GM-CSF (0.35 μg) + empty pBudCE4.1 (1.0 μg); D) pNGVL1/GM-CSF (0.35 μg) + pBudCE4.1/Ii-RGC(x3) (0.325 μg) and empty pBudCE4.1 (0.675 μg); E) pNGVL1/GM-CSF (0.35 μg) + pBudCE4.1/Ii-RGC(x3) (1.0 μg). Medium only is represented by open bar. p18 peptide is represented by dotted bar, or p18-I10 peptide is represented by black bar.

2) Define the *in vivo* efficacy of MHC class II+/Ii-phenotype immunotherapy and protection the growth of metastatic tumor cells. Optimize the frequency of immunizing schedule and define the optimal doses of plasmids that induce MHC class II+/Ii- phenotype *(from 2nd and 3rd year of work)*

a. MHC class II+/Ii- phenotype therapy generates an immune response that protects mice from challenge with the same tumor cells. The work planned in Task 3 has been accomplished as part of a collaboration with Dr. Hillman. Those studies characterized the activities of CD4+ T helper cells and CD8+ CTL that were induced by intra-tumoral injection to express the MHC class II+/Ii- phenotype. Her team has shown that a low dose of radiation plus all vectors of pCIITA, pIFN-γ, pIi-RGC and pIL-2 induced the strongest CD4+ T helper and CD8+ CTL activity. They have further shown that induction of both MHC class II and class I molecules plus the inhibition of the Ii protein (to produce the MHC class I+/II+/Ii- phenotype) is necessary for the optimal therapeutic efficacy against challenge with RM-9 prostate tumor cells (produced the

highest percentage of cured mice). The cured mice also produced a potent and long-lasting immune response that protected the mice from re-challenge with RM-9 cells in a distal location from the original challenge site but did not protect mice from the challenge with other tumor cells. This result clearly demonstrates that the MHC class I+/II+/Ii- phenotype induces a potent tumor-specific immune response that can eliminate tumor cells (etc. metastasis tumor cells) of same antigenicity (no mutation or lost immunogenecity). Dr. Hillman and her colleagues have also done histological evaluation of tumor cells by MHC class II+/Ii- immunotherapy. They found that irradiation plus intratumoral therapy using all vectors led to tumor cell destruction, while either irradiation or all vector intratumoral therapy alone was not fully effective [1].

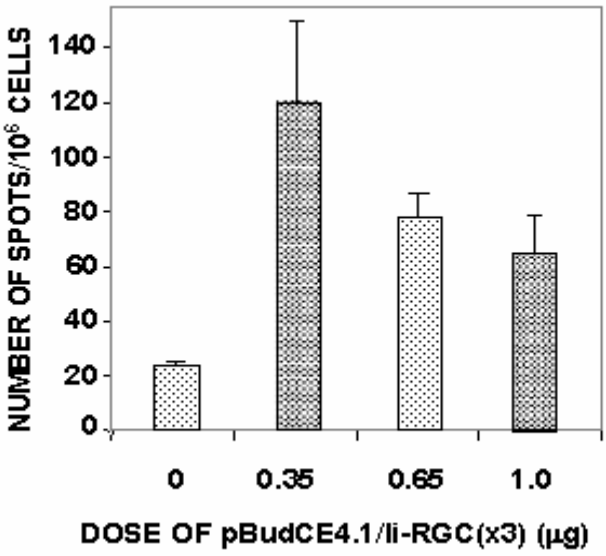

Figure 7. The correlation between concentration of Ii-RGC and IFN-γ production to P18 stimulation. The doses of gp120 and GM-CSF plasmids are constant in all groups and the in lower dose of pBudCE4.1/Ii-RGC(x3) group, the empty plasmid was added to keep total DNA at same amount. One can clearly see from this figure that the IFN-γ produced by p18 stimulation is well correlated to the dose of pBudCE4.1/Ii-RGC(x3) used.

b. Optimization of the frequency, doses of gene therapy vectors. Dr. Hillman and her colleagues have evaluated the influence of frequency of intratumor injection on therapeutic efficiency and they found that more frequent injections did not produce a better therapeutic outcome (4 consecutive injections where found to be sufficient). Using either a plasmid IL-2 or a recombinant adenovirus containing IL-2 gene, Dr. Hillman has shown that a sub-therapeutic dose of IL-2, when given with MHC class II+/Ii- phenotype vaccine, was sufficient to generate a potent anti-tumor immune response. The dose of Ii inhibition plasmid (pIi-RGC) has been evaluated in a DNA vaccine study. The study showed that the low dose of Ii-RGC(x3) plasmids produced better gp120 DNA vaccine efficacy. The higher dose did not generate more potent DNA vaccine efficacy; instead, it lead to a reduced efficacy, possible due to promoter competition (**Figure 7**) [2]. This result indicated indirectly that the doses of plasmids for the induction of the required phenotype should be kept at the lowest level, that is, at the minimum level which effectively induces the desired phenotype in tumor cells.

The above experiments allowed us to evaluate and optimize the parameters for injection frequency, injection schedules, and doses of plasmids for the induction of phenotype and immune response. These parameters provide useful guides for a future prostate cancer clinical trial using MHC class I+/II+/Ii- phenotype induction immunotherapy. These results have clearly indicated that MHC class I+/II+/Ii- phenotype induction generates a potent, long-lasting and tumor-specific immune response which effectively eliminates the same tumor cell line when re-challenged. However, this immune response did not protect mice from challenge using other kinds of tumor cells, indicating that the response is tumor specific and that metastasized tumor

cells will be eliminated if they do not change their antigenecity.

3. Perform the toxicology studies including the bio-distribution of therapeutic reagents for a possible prostate cancer clinical trial (*from 3rd year work*).

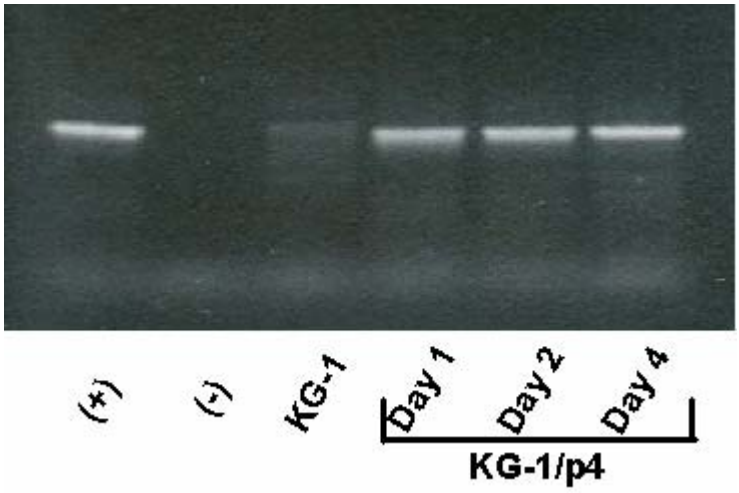

Figure 8. Time cause of Ii-RNAi plasmid in cells. KG-1 cells were transfected with pBudCE4.1/p4 plasmid with gene gun (1 μg/10^6/shoot). At different time points, cells were washed three times and cellular DNA was extracted. The cellular DNA was used as template in PCR assay. Positive control uses 15 ng of pBudCE4.1/p4 as template and negative control uses water (used for dissolve cellular DNA). KG-1 is cells have no transfection. KG-1/p4 is KG-1 cells transfected with pBudCE4.1/p4.

a. Duration of Ii-RNAi construct in transfected cells in vitro. At this point in time, we do not foresee significant changes in the long-term goal: prostate cancer immunotherapy. However, we feel that in the clinic, an intratumoral injection might be more difficult to accomplish than MHC class I+/II+/Ii- phenotype cell-based immunotherapy. Modification of surgically removed tumor cells *in vitro* and infusion of the modified cells into patients is easier to perform and has less risk than intratumor injection. We have performed toxicology studies, such as plasmid transfected cell bio-distribution *in vivo*. First, we tested the Ii-RNAi plasmid duration in KG-1 cells (ATCC). KG-1 cells were transfected with pBudCE4.1/p4 plasmid by gene gun mediated DNA transfection. Briefly, 10^6 KG-1 cells in 20 μl medium were smeared onto a 10-cm tissue culture dish in 0.6-0.8 cm diameter circle. The cells were then subjected to a gene gun shooting with 1 μg of pBudCE4.1/p4 plasmid. Three gene gun shootings were performed in one tissue culture dish and then 10 ml of culture medium was added. The dishes were cultured for 1, 2, or 4 days. The cells were harvested and total DNA was extracted with QIAmp mini DNA kit (Qiagen) according to the manufacturers' instruction. PCR were then performed with extracted DNA as a template using the oligonucleotides that hybridize to the EF-1a promoter sequence and BGH poly A sequence. The PCR reactions have shown that the Ii-RNA plasmid can be detected in the cells 4 days after transfection (**Figure 8).**

b. Bio-distribution of plasmid-transfected tumor cells in vivo. We injected pBudCE4.1/p4-transfected KG-1 cells into BALB/c mice. The cells were transfected as described above. The transfected cells were cultured for 24 hours and then harvested, washed and irradiated with 3500 rad. Mice were injected subcutaneously on the left leg with $4x10^6$ KG-1/p4 cells in 50 μl PBS. At day 2 and day 8 after cell injection, mice (2 mice at each time point) were sacrificed and organs taken (lymph nodes, kidney, liver, spleen, lung, heart, brain, and muscle at injection site). Total DNA was extracted from the tissues with QIAmp mini DNA kit (Qiagen) according to the manufacturer's instruction. PCR was then performed with 100 ng of organ DNA as template. From **Figure 9**, one can see that all PCR reactions were negative except for trace amounts of

non-specific PCR background (un-immunized mice had the same background). This DNA bio-distribution study indicates that the quantity of DNA in the organs is undetectable by PCR reaction.

This result suggests that transfecting cells *in vitro* and then immunizing mice with the transfected cells is a safe and possibly a more efficient therapy than directly injecting DNA into the *in vivo* tumor. The quantity of DNA needed is much lower for in *vitro* transfection (3 μg if all gold-microparticles were injected into cells versus 160 μg for intratumor injection). The potency of cell-based immunotherapy, however, is similar or possibly higher than intratumoral injection therapy.

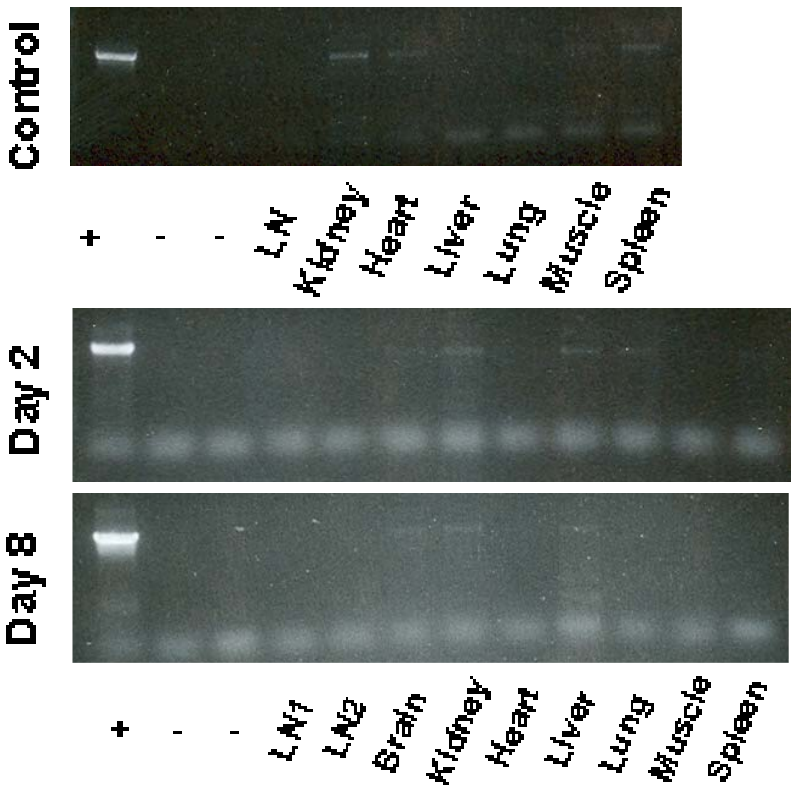

Figure 9. Bio-distribution of plasmid that transfected into tumor cells and then injected into mice. pBudCE4.1/p4-transfeted KG-1 cells ($4x10^6$ were injected into left leg of BALB/c mice in 50 μl volume. At different time points, organs were taken and DNA were extracted. pBudCE4.1/p4 was monitored by PCR with two DNA primers hybridize to promoter and poly A sequences in plasmid. Positive is 15 ng of plasmid and negative controls are no template or H_2O as template. LN1 is injection side inguinal lymph node and LN2 is other side inguinal lymph

c. Constructing double Ii-RNAi and determining the activity of double Ii-RNAi construct and the activities of Ii-RNAi constructs with different promoters in prostate cancer cell line.

i. Constructing double Ii-RNAi construct. As we indicated in our previous reports, the combined use of Ii-RNAi constructs that target a different site of Ii mRNA produced better Ii inhibition in Raji cells (**Figure 4**). In order to optimize the Ii suppression reagent for clinical use (to reduce the total DNA to be used), we have spent some time designing a double Ii-RNAi construct. To construct the double Ii-RNAi construct, two oligonucleotides were synthesized: one hybridizes to the EF-1a promoter sequence and the other hybridizes to the BGH poly A signal sequence. Two endonuclease sites, Spe1 and BamH1, were designed on two oligonucelotides, respectively. The PCR product was sequenced to confirm the existence of the Ii-RNAi, p7 sequence. The PCR product was then digested with Spe1 and BamH1 and the digested PCR product ligated into a pBudCE4.1/p4 that was digested with Spe1 and BamH1 (to eliminate the CMV promoter and some of the cloning sites). The resulting colonies were checked by Spe1 and BamH1 digestion and one of them was produced in a large quantity.

ii. Testing the activity of double Ii-RNAi in PC-3 prostate cancer cells. We have tested the activity of double Ii-RNAi constructs in PC-3 cells (ATCC). PC-3 prostate cells were tansfected with EF-1a/P7 and EF-1a/p4/p7 plasmids using lipofectamine2000 (Qiagen). 36 to 48 hours after transfection, cells were harvested, washed, and stained with the anti-Ii antibody, LN2 (BD Pharmingen). As indicated in **Figure 10**, the activity of the double Ii-RNAi construct was not significantly more active than the single Ii-RNAi construct (compare EF-1a/p7 with EF-1a/p4/p7). The reason for no enhancement of the double Ii-RNAi compared to single Ii-RNAi is not known. According to our experience (and that of others), construction of two or more expression cassettes in one plasmid usually results in enhanced expression of the plasmid. For example, the activity of Ii-RGC(x3) is more active than Ii-RGC (see our attached paper). The orientation of the expression cassettes might influence the activity of the expression cassettes on the same plasmid (Feng He, personal communication). Since the use of a combination of two different Ii-RNAi constructs gave better Ii inhibition, we will use a combination of two different plasmids, p4 and p7 in a future clinical trial.

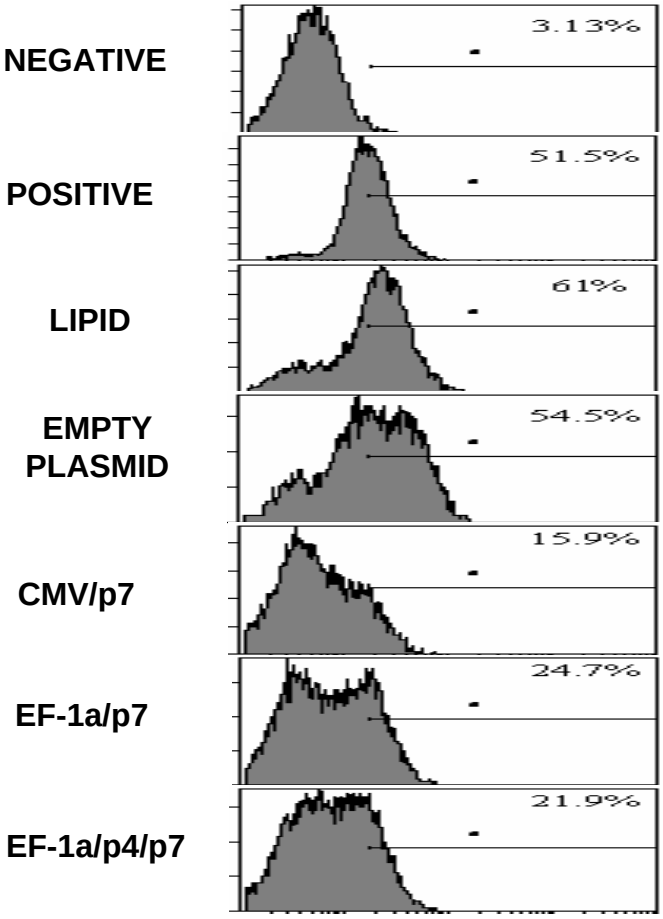

Figure 10. Ii inhibition by different Ii-RNAi constructs in PC-3 prostate cancer cells. PC-3 cells ($3x10^5$ cells were plated in 6-well plates one day before transfection. Transfections were performed with lipo- fectamine2000 according to manufac-turer's instruction. Two days after transfection, cells were harvested, washed, and stained with anti-human Ii antibody. Negative and positive are not treated cells stained without or with anti-Ii antibody. LIPID is transfection without plasmid.

iii. Determine the activity of Ii-RNAi constructs driven by different promoters. In a previous annual report, we have shown that the EF-1a promoter is more active in AML cells than is the CMV promoter. The result is consistent with the study by Salmon *et al*. who have shown that the EF-1a promoter is more active than the CMV promoter in CD34+ hematopoietic cells [3]. To elucidate whether this phenomenon is the same for prostate cancer cells, or if this phenomenon is cell type-specific, we have compared the activities of the Ii-RNAi constructs driven by a CMV promoter and an EF-1a promoter in PC-3 prostate cells. PC-3 cells were transfected with CMV/p7 and EF-1a/P7 plasmids by the lipofectamine2000 (Qiagen) method. The result demonstrates that in PC-3 cells, the CMV promoter is more active than EF-1a promoter (compare CMV/p7 with EF-1a/p7) (**Figure 10**). This result indicates that the activity of

the promoter is cell type-specific and suggests that the promoter plays an important role in the efficacy of genetic vaccines such as DNA vaccines as well as genetically modified tumor cell vaccines. We will use Ii-RNAi constructs driven by a CMV promoter in a future prostate cancer clinical trial.

iv. Heterogeneity of prostate cell lines. The PC-3 cell line is an MHC class II-/Ii+ prostate cancer cell line (data not shown). In our early study, we have shown that in fresh colon carcinoma samples, MHC class II expression did not correlate with the malignancy of tumors. However, Ii expression strongly correlated with the malignancy of colon carcinoma [4]. In that study, many colon cancer cells are MHC class II negative while the Ii remains positive. This result suggests that Ii expression may help tumor cells escape from immune surveillance by preventing endogenous tumor antigens from presentation to CD4+ T helpers by MHC class II molecules. The mechanisms for converting to a MHC class II-/Ii+ phenotype in tumor cells (colon cancer cells and PC-3 prostate cancer cells) remains unknown but the results strongly indicate that an Ii suppression method is an elegant method to create a potent tumor cell immunotherapy. Transfection of PC-3 prostate cancer cells with CIITA induced little MHC class II expression (<4% of cells weak positive) while transfection of another prostate cancer cell line, LNcap (ATCC), with CIITA induced good MHC class II expression (50% of cells MHC class II positive, data not shown). Parental LNcap prostate cells are also MHC class II-/Ii+ cells (data not shown). DU-145 cells are MHC class II-/Ii- and the MHC class II and Ii can be induced by transfecting CIIT gene (**Figure 2**). These results indicate that these two prostate cell lines are heterogeneous in terms of MHC class II induction by CIITA. Three prostate cells use different mechanisms to escape from immune surveillance. CIITA is driven by a CMV promoter, indicating that the CMV promoter is active in DU-145 and LNcap cells. Collectively, our results indicate that the CMV promoter is a very active promoter in all three prostate cell lines.

KEY RESEARCH ACCOMPLISHMENTS

1. We have generated potent Ii suppression reagents and optimized the combination use of human Ii-RNAi constructs. We have defined the influence of the promoter in the activity of Ii-RNAi. Our results indicated that two elements are important for the activity of an Ii-RNAi construct: a) Identification of the Ii-RNAi sequence that targets the specific site of Ii mRNA and b) Selecting the promoter that is most active in that cell line. We have demonstrated that the CMV promoter is most active in prostate cancer cells.
2. We have tested the activity of our Ii-RNAi constructs in primary AML cells. It is critical for a clinical trial that the human Ii-RNAi constructs used are active in the primary tumor cell samples of a given tumor.
3. Ii suppression and the resulting enhancement of a DNA vaccine has been tested and confirmed using a gp120 DNA vaccine model. We have obtained a five-fold enhancement of a gp120 DNA vaccine by Ii suppression. This result further confirms that Ii suppression enhances the endogenous antigen presentation by MHC class II molecules without interrupting MHC class I antigen presentation, thus enhancing the potency of a tumor cell vaccine and a DNA vaccine.
4. We have shown that an anti-tumor immune response has been strongly induced by intratumoral induction of the MHC class I+/II+/Ii- phenotype. This immune response is tumor specific and effectively protects mice from re-challenge of the same tumor cells but not other tumor cells. These results indicate that the immune response induced by tumor vaccine cells

expressing MHC class I+/II+/Ii- phenotype can eliminate tumors metastases if their antigenicity and immunogenicity are not changed from the primary tumor.
5. The frequency of injections and the doses of plasmids for induction of the MHC class I+/II+/Ii- phenotype and the dose of IL-2 plasmid have been evaluated. The results indicate that they should be kept at the minimum level that effectively induces the target phenotype. A low dose of IL-2 is sufficient for the induction of a potent immune response by MHC class I+/II+/Ii- phenotype.
6. The duration of plasmid in cells *in vitro* and the *in vivo* bio-distribution of DNA transfected into cells has been evaluated. After injection of plasmid transfected tumor vaccine cells, the DNA level in organs is undetectable by PCR. Our results indicate that genetically modified tumor cell-based immunotherapy should be quite safe.
7. We have found that DU-145, PC-3, and LNcap prostate cell lines are heterogeneous. The heterogeneity of prostate cancer cell lines indicates that the cell lines are useful tools to generate reagents in the laboratory. However, a cell line only represents a single cancer lineage *in vivo*. PC-3 and LNcap cells are of MHC class II-/Ii+ phenotype. This phenomenon suggests that Ii may play a role in prostate cancer generation and progression. This phenomenon also indicates that our Ii suppression method is an elegant method to generate potent prostate cancer immunotherapy.

REPORTABLE OUTCOMES

Presentations

1. "Generation of the MHC class II+/Ii- phenotype on tumor cells by Ii-RGC or Ii-RNAi leads to a potent tumor cell immunotherapy." Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. American Association for Cancer Research. Anaheim, CA, April 16-20, 2005

2. "Forcing tumor cells to actively present MHC class II-restricted endogenous tumor antigens by inhibiting MHC class II-associated invariant chain expression by Ii-RGC and Ii-RNAi." Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. Keystone Symposium "Basic Aspects of Tumor Immunology.". Keystone CO, March 19, 2005

3. "Forcing tumor cells to actively present MHC class II-restricted endogenous tumor antigens by inhibiting MHC class II-associated invariant chain expression by Ii-RGC and Ii-RNAi." Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. Keystone Symposium "Basic Aspects of Tumor Immunology.". Keystone CO, March 19, 2005

4. "Potent Therapeutic Cancer Vaccine Generated by Tumor Irradiation and Genetic Induction of MHC class I+/class II+/Ii- Tumor Phenotype." Gilda Hillman1, Minzhen Xu2, Mingxin Che1, Eric Von Hofe2, Asad Abbas1 and Yu Wang1. Keystone Tumor Immunology (C3) Mar 19 - Mar 24, 2005.

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1. Xu M, Lu X, Kallinteris NL, Wang Y, Wu S, von Hofe E, Gulfo J, Humphreys RE, Hillman GG. Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules. Curr Opin Mol Ther. 2004; 6:160-5.
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4. Lu X, Wu S, Blackwell CE, Humphreys RE, von Hofe E, Xu M. Suppression of major histocompatibility complex class II-associated invariant chain enhances the potency of an HIV gp120 DNA vaccine. *Immunology*. 120:207-16 (2007). 2006 Nov 20; [Epub Nov 2006].

Personnel receiving pay for research effort

Robert Humphreys
Minzhen Xu
Eric von Hofe
Xueqing Lu
Shuzhen Wu
Catherine Blackwell
Douglas Powell
John Zinckgraf
Nikoletta Kallinteris
Margaret Sposato

CONCLUSIONS

Importance and implications. First, we have successfully constructed and validated both murine and human reagents for inhibition of Ii-expression. Generating potent Ii-RNAi constructs, optimizing the combined use of these constructs, and defining the most active promoter to drive the expression of Ii-RNAi in a given cell line or certain type of fresh tumor cells are the necessary steps to ensure generation of the MHC class I+/II+/Ii- phenotype in prostate cancer cells. Second, we have clearly shown that both CD4+ T helper and CD8+ CTL activation was strongly induced by intra-tumor induction of the MHC class I+/II+/I- phenotype, generating a potent, tumor-specific, and long-term anti-tumor immune response. Such MHC class I+/II+/Ii- induced tumor-specific immune responses can eliminate tumor cells of the same antigenicity.

These results indicate that metastatic tumor cells can be eliminated if they have not changed their antigenicity or immunogenicity. Thirdly, the importance of frequency of intratumoral injections and the doses of plasmids has been established. We also evaluated the bio-distribution of plasmid transfected tumor cells *in vivo*. These achievements continue to pave the way to achieve our major long-term goal – curative immunotherapy of prostate cancer. We expect this therapy to far exceed the efficacy of other DNA vaccines, dendritic cell vaccines, dendritic/tumor fusions, or dendritic/tumor extracts. Lastly, our study has also revealed that two prostate cell lines are of MHC class II-/Ii+ phenotype, which implicates the importance of Ii expression preventing an immune response. This observation provides additional correlative evidence that Ii inhibition is an elegant method to generate a potent prostate cancer immunotherapy. This is the only technology that can force living tumor cells to actively present endogenous tumor antigens to the immune system.

Changes in future work to better address the problem. At this point in time, we do not foresee significant changes in the long-term goal: prostate cancer immunotherapy. However, we feel that *in vitro* modification of surgically removed prostate cancer cells and re-infusion of these modified tumor cell back into patients is simpler and less intrusive than intra-tumor injection. Prostate cell lines are heterogeneous in terms of MHC class II expression. We have determined the immunizing doses, schedules and the doses of IL-2 and other plasmids for optimal induction of the MHC class I+/II+/Ii- phenotype. All of this information provides useful parameters for a future prostate cancer immunotherapy clinical trial. All reagents are ready for such a trial.

Evaluation of the knowledge as a scientific or medical product. The experiments under this grant generated Ii-RNAi constructs active in both prostate cancer lines and fresh tumor cells, paving the way for the clinical trials for tumor immunotherapies including prostate cancer. Immunotherapy by generation of the MHC class I+/II+/Ii- phenotype appears to be a robust method with good potential for immunotherapy of prostate cancer as well as for other types of cancer.

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APPENDIX

PRESENTATIONS

GENERATION OF THE MHC CLASS II+/II- PHENOTYPE ON TUMOR CELLS BY II-RGC OR II-RNAI LEADS TO A POTENT TUMOR CELL IMMUNOTHERAPHY.

Xueqing Lu, Nikoletta Kallinteris, Shuzhen Wu, Robert Humphreys, Eric Von Hofe, Minzhen Xu. Antigen Express, Worcester, MA 01606

RNAi is a potent method to inhibit specific gene expression. This method has been evaluated as a potential tool to treat cancer, for example, to specifically inhibit oncogene expression. The biggest challenge for using RNAi to inhibit oncogene expression is the requirement for in vivo transfection of alltumor cells permamently by RNAi constructs. We have developed II-RGC and Ii-RNAi methods to effectively suppress in tumor cells the expression of invariant chain (Ii protein) that normally blocks antigenic peptide binding site of MHC class II molecules during synthesis in the endoplasmic reticulum (ER). In such genetically engineered tumor cells, both MHC class I and class II molecules pick up endogenous antigenic peptides (including tumor antigens) in the ER. Simultaneous presentation of these tumor antigens by both MHC class I and class II molecules to both CD4+ and CD8+ T cells generates a robust and long –lasting anti-tumor immune response in mice. An advantage of this strategy is that we do not need to transfect all tumor cells permanently. Transfecting only a fraction of the total tumor cells transiently is sufficient to induce an anti-tumor immune response. Our novel method forces tumor cells to actively present their tumor antigens and thus has the potential to lead to a feasible and potent tumor cell immunotherapy. We have now generated human Ii-RNAi constructs that effectively inhibit Ii-expression in Raji lymphoma cells and 293 kidney cells. Ii inhibition by active Ii-RNAi constructs reached 95% in Raji cells while a combination of different Ii-RNAi constructs targeting different positions of the ii gene has a synergistic effect on Ii inhibition, reaching about 99% Ii suppression. Because Ii is monomorphic, one Ii-RNAi construct(s) may be sufficient for all patients regardless of their HLA-DR allele. The generation of these active Ii-RNAi constructs provides suitable reagents paving the way for human cancer clinical trials.

TUMOR IRRADIATION POTENTIATES GENE-MEDIATED IMMUNOTHERAPY FOR INDUCTION OF *A* CURATIVE CANCER VACCINE.

Hillman GG[1], Xu M[2], Che M[1], Von Hofe E[2], Lu X[2], Forman JD[1] and Wang Y[1]. [1]Department of Radiation Oncology and Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, United States and [2]Antigen Express, Inc.,Generex Biotechnology Corp., Worcester, MA 01606, United States.

Objective: We have shown that tumor irradiation preceding the transfection of genes into tumors, to up-regulate MHC class I and class II molecules and inhibit invariant chain (Ii), induces a potent anti-tumor immune response in murine RM-9 prostate carcinoma syngeneic to C57BL/6

mice. Such cancer cells become antigen-presenting cells (APCs) that present both class I and class II endogenous tumor antigens, triggering a potent T-helper response essential for robust cytotoxic T cell activity (CTL). Inhibition of Ii protein increases presentation of endogenous tumor peptides by class II molecules to helper T cells. The mechanism by which tumor irradiation enhances the efficacy of gene therapy for induction of cancer vaccine was investigated.

Materials and Methods: To induce, *in situ,* the MHC class I+/class II+/Ii- phenotype, we used cDNA plasmids containing genes for interferon gamma (pIFN-g) to upregulate MHC class I, MHC class II transactivator (pCIITA) to upregulate MHC class II, an Ii reverse gene construct (pIi-RGC) to suppress Ii, and a subtherapeutic dose of adjuvant IL-2 (pIL-2). Established RM-9 tumors were treated with 8 Gy photon radiation followed by 4 days of intratumoral injections with a mixture of pCIITA + pIFN-g + pIi-RGC + pIL-2 plasmids. Viability of cells isolated from treated tumors at different time points was assessed by colony formation assay. Tumor destruction was assessed on tumor sections by histology and TUNEL assay.

Results: An optimal and specific anti-tumor response is achieved in more than 50% of the mice when, following radiation, tumor nodules are treated with the four pIFN-g, pCIITA, pIi-RGC and pIL-2 plasmids. Mice responding with complete tumor regression rejected tumor rechallenge and demonstrated tumor-specific CTLs. Such therapeutic effect was achieved only when tumor irradiation preceded gene therapy and when the combination of the four plasmids were injected intratumorally to convert tumors to MHC class I+/class II+/Ii- phenotype. Omission of radiation or either one of the plasmids decreased the tumor response and giving gene therapy prior to radiation was not as effective. We demonstrated further that both CD4+ helper T cells and CD8+ cytotoxic T cells are essential for induction of an anti-tumor response because in vivo depletion of either subset abrogated the response. Apoptosis was documented in tumor sections by TUNEL assay as early as one day after radiation, at the time gene therapy was initiated. Radiation caused significant debulking of the tumors in situ as demonstrated by significant colony formation inhibition of cells isolated from tumors at early time points between days 1-13 after radiation treatment. Complete tumor destruction by combined radiation and gene therapy was determined by lack of colony formation of cells isolated from these tumors and by histological observation. Histological analysis of tumor sections shows that tumor irradiation combined with plasmids causes extensive destruction of tumor cells, large areas of apoptosis and necrosis associated with a massive infiltration of lymphocytes and PMN. This effect is seen 1 day after gene therapy and persists for several days while tumor regrowth follows the initial focal apoptosis and necrosis observed after radiation or plasmids alone. We further showed that radiation potentiates the genetic modification of tumor cells by increasing both the level and duration of expression of transfected genes.

Conclusions: Our findings suggest that radiation potentiates gene therapy by causing tumor debulking, increasing gene transfection and the permeability of tumors to infiltration of inflammatory cells. These data emphasize the efficacy of tumor irradiation preceding gene therapy to modify tumor cells in situ into a MHC class I+/class II+/Ii- phenotype converting these cells into a potent therapeutic cancer vaccine.

FORCING TUMOR CELLS TO ACTIVELY PRESENT MHC CLASS II-RESTRICTED ENDOGENOUS TUMOR ANTIGENS BY INHIBITING MHC CLASS II-ASSOCIATED INVARIANT CHAIN EXPRESSION BY II-RGC AND II-RNAI

Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu

Antigen Express, Inc. 100 Barber Avenue, Worcester, MA 01606

Immunological cure of tumors depends on initiating both CTL and T helper cell responses to endogenous tumor antigens. In contrast to other tumor immunotherapies, we have developed a novel approach that forces tumor cells actively present endogenous tumor antigens to stimulate CD4+ T helper cells by converting tumor cells into MHC class II+/Ii- phenotype. Using antisense methods previously, we suppressed expression of the invariant chain (Ii protein) that normally blocks the antigenic peptide binding site of MHC class II molecules during synthesis in the endoplasmic reticulum (ER). In such genetically engineered tumor cells, the MHC class II molecules pick up endogenous antigenic peptides (including tumor antigens), which have been transported into the ER for binding to MHC class I molecules. The simultaneous presentation of these tumor antigens by both MHC class I and II molecules generates a robust and long-lasting anti-tumor immune response. Injecting murine tumors with genes to induce MHC class II and suppress Ii protein expression, results in the cure of a significant number of animals with renal and prostate tumors. An advantage of this strategy is that we do not need to transfect all tumor cells permanently. Transfecting only a fraction of the total tumor cells transiently is sufficient to induce a complete anti-tumor immune response. We have now developed human Ii-RNAi constructs that efficiently inhibit Ii expression in human tumor cell lines including Raji lymphoma cells and 293 kidney cells. Since Ii is monomorphic, one Ii-RNAi construct is suitable for all patients with different HLA-DR alleles. The generation of active human Ii-RNA paves the way to generate MHC class II+/Ii- human tumor cells for clinical trial.

POTENT THERAPEUTIC CANCER VACCINE GENERATED BY TUMOR IRRADIATION AND GENETIC INDUCTION OF MHC CLASS I+/CLASS II+/II- TUMOR PHENOTYPE.

Gilda Hillman[1], Minzhen Xu[2], Mingxin Che[1], Eric Von Hofe[2], Asad Abbas[1] and Yu Wang[1].
[1]Department of Radiation Oncology and Pathology, Karmanos Cancer Institute, WSU, Detroit, MI 48201, [2]Antigen Express, Inc, Generex Biotechnology Corp, Worcester, MA 01606.
We showed that *in situ* genetic modification of murine RM-9 prostate tumor cells, to express MHC class I and class II molecules and suppress MHC class II associated invariant chain Ii, converts those cells into a cancer vaccine. Gene therapy was delivered intratumorally using plasmids coding for IFN-γ, CIITA, an Ii reverse gene construct (Ii-RGC), and a low IL-2 plasmid dose. Complete tumor regressions, and induction of specific anti-tumor immune response, were obtained only when gene therapy was preceded, one day before, by 8Gy tumor irradiation. By selective in vivo depletion of T cell subsets, we demonstrate further that both CD4+ helper T cells and CD8+ cytotoxic T cells are essential for induction of a potent anti-tumor response. *In vitro* colony assays of cells isolated from tumors, 1 day after radiation, show 60% inhibition in division ability, thus radiation causes tumor debulking and increases the probability of cell transfection. Histology of tumors treated with radiation and gene therapy shows complete tumor destruction and that radiation increases the permeability of tumors to infiltration of inflammatory cells. Radiation enhances gene therapy by causing tumor debulking and increasing tumor permeability.

PAPERS

Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules

Minzhen Xu[1], Xueqing Lu[1], Nikoletta L Kallinteris[1], Yu Wang[2], Shuzhen Wu[1], Eric von Hofe[1], Joseph V Gulfo[1], Robert E Humphreys[1]* & Gilda G Hillman[2]

Addresses
[1]Antigen Express Inc
100 Barber Avenue
Worcester
MA 01606
USA
Email: humphreys3@aol.com

[2]Wayne State University School of Medicine
Barbara Ann Karmanos Cancer Institute
Department of Radiation Oncology
4100 John R
Detroit
MI 48201
USA

*To whom correspondence should be addressed

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Ii protein suppression is a promising antisense drug-based therapy that dramatically enhances the immunogenicity of tumor cell major histocompatibility complex class II-presented antigenic epitopes. The strength of this approach is that the antisense only needs to be transiently effective in a fraction of the tumor cells. The systemic antitumor immune response generated subsequently eradicates both directly treated cells and distant tumor deposits. The drugs and mechanisms of this therapy are considered, in addition to practical developmental questions.

Keywords Cancer vaccine, Ii antisense, immunotherapy, major histocompatibility complex class II, T-helper cells

Introduction

Thwarting a tumor's escape from immune surveillance

Tumors escape the host immune surveillance, which normally prevents evolution of a malignancy by blocking presentation of immunogenic tumor-associated antigenic epitopes. Additionally, tumors suppress the antitumor immune response by developing active immunosuppressive mechanisms against dominant T-cell-recognized antigenic epitopes of the tumor.

Protein Ii can be inhibited in two ways against these tumor growth-favoring mechanisms. Presentation of tumor-associated antigenic epitopes is enhanced by rearranging the antigen-processing pathway to allow major histocompatibility complex (MHC) class II presentation of tumor self-proteins to T-helper (Th) cells. This method also favors presentation of cryptic and subdominant epitopes to which immunosuppression had never developed previously. The approach can even cure mice of an aggressive prostate tumor that is poorly immunogenic. The biological mechanism of this therapy and the steps taken to bring it to the clinic are reviewed.

Overview of mechanism and therapeutic potential

Normally, antigenic epitopes of cellular self-proteins are transported from the cytoplasm into the endoplasmic reticulum for binding to newly synthesized MHC class I molecules, but not to MHC class II molecules, which are blocked by the Ii protein. The Ii protein is effectively suppressed with antisense oligonucleotides or reverse gene constructs. This process can be 'rearranged' in the endoplasmic reticulum of such Ii-suppressed tumor cells, to permit MHC class II molecules to bind tumor-associated antigenic epitopes for subsequent presentation to self-surveying T-cells. New epitopes presented by MHC class II molecules stimulate Th cells to enhance the activity of tumor-specific cytotoxic T-lymphocytes (CTLs) and create long-lasting antitumor immunological memory. The repertoire of MHC class II epitopes is also expanded to include 'cryptic epitopes', to which a cancer patient's immune system has never been exposed. The presentation of such epitopes by MHC class II molecules can reverse immunological tolerance to the tumor and cure an established tumor, at least in mice. While this antisense drug is directly therapeutic when injected into tumors that are either naturally MHC class II-positive or made such by co-transfection of genes for MHC class II transactivator (CIITA) or interferon (IFN)γ, it also enables many additional therapies. Ii suppression can enhance various DNA vaccines for tumor or infectious disease antigens. In addition, novel peptide therapeutics can be mined from the repertoire of induced MHC class II epitopes found in MHC class II+/Ii- cultured cells of tumors or antigen-presenting cells (APCs) loaded with antigens relevant to autoimmune disease. In short, antisense-induced Ii suppression enables a wide range of antigenic epitope diagnostics and therapeutics.

Evidence for mechanisms

Antigen processing and presentation pathways potentially blocked during tumorigenesis

All nucleated cells express MHC class I molecules. At the time of their synthesis, these molecules bind antigenic peptides derived from cytoplasmic proteins, which are processed into peptides by proteosomes and transported by the transporter of antigenic peptides (TAP) into the endoplasmic reticulum [1,2]. β_2-Microglobulin binds to MHC class I molecules, locking them into a conformation that tightly holds the antigenic peptide for the duration of its presentation at the cell surface [3].

MHC class II molecules are normally expressed on professional APCs, such as dendritic cells (DCs), macrophages and B-cells, to induce a CD4+ Th cell response. Such Th cells induce DCs to a stage of activation defined as 'licensing', which stimulates and activates specific CTLs [4•,5•,6].

Normally, MHC class II molecules are blocked by the Ii protein at the time of their synthesis, and receive exogenous antigen selected by the APCs in a post-Golgi compartment. There, certain proteases that cleave the foreign antigen also

cleave and release Ii fragments from the MHC class II molecules in a concerted exchange process, during which antigenic peptide is inserted into the binding site of class II molecules [7,8]. This pathway prevents expression of endogenous peptides by MHC class II molecules in tumor cells. Interestingly, an increase in Ii in hairy leukemic cells and the inverse correlation of Ii expression and tumor-infiltrating lymphocytes in human colon carcinomas suggest an immunosuppressive role for Ii in such malignancies [9,10]. MHC class II molecules appear to influence the antitumor response [11-13,14•]. The absence of, or defects in these antigen processing and presentation pathways have been individually reported to promote non-recognition of tumors.

Tumor defense by immunosuppression

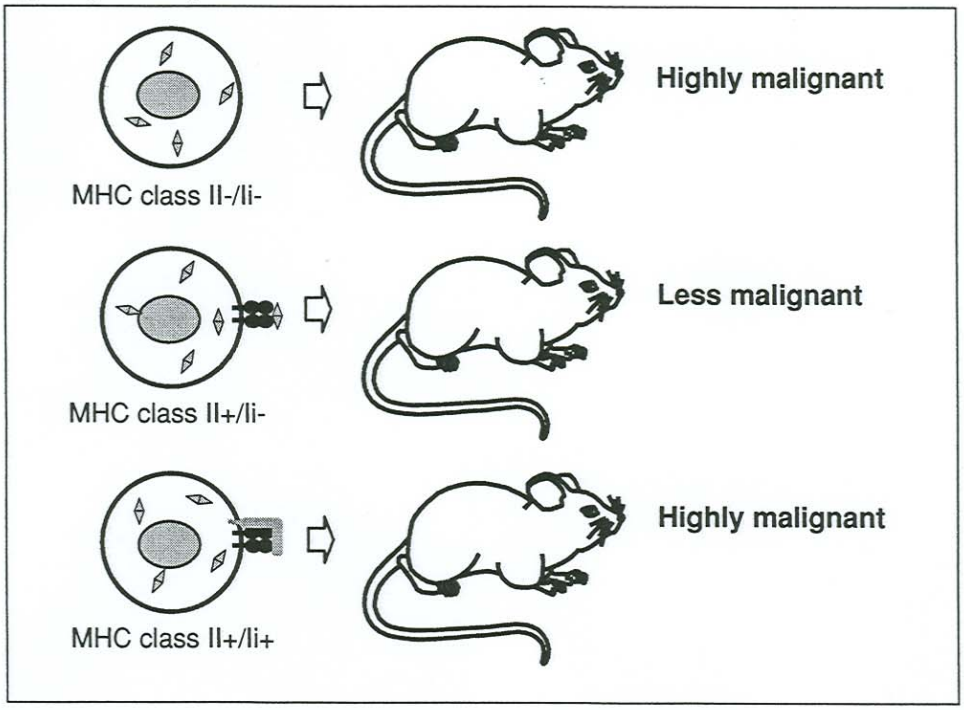
Dominant epitopes within a population of tumor-associated antigenic epitopes often induce suppression, which shuts off all antitumor immune responses. This immunosuppression is sometimes associated with the T-cell subset Th3 CD4+/CD25+ immunoregulatory cells [15••,16,17••,18], which secrete interleukin (IL)-10 and transforming growth factor (TGF)β. This is in contrast to the IFNγ-secreting Th1 cells, a Th subset that promotes CTL responses [19•,20-22].

One can hypothesize that by downregulating expression of the Ii protein, a much larger repertoire of MHC class II-restricted epitopes is presented, including cryptic epitopes, which have never been seen before by the immune system. The immune system also recognizes newly exposed, lower-affinity Th cell-recognized epitopes, to which tolerance has never been developed. The response to both types of such non-immunosuppressed epitopes leads to a robust Th1 response, stimulating tumor-specific CTLs and providing long-term immunological memory.

Pioneering experiments

Suzanne Ostrand-Rosenberg and colleagues discovered the principle that MHC class II+/Ii- tumor cells, made MHC class II-positive by transfection of genes for syngeneic MHC class II α and β chains, and not expressing Ii protein, are potent anticancer vaccines [23-25]. These researchers characterized the immunological mechanisms in detail (Figure 1). Mice vaccinated with MHC class II gene-transfected Sal 1 sarcoma cells rejected subsequent challenges with the parental MHC class I+/MHC class II- Sal I cells [23]. However, supratransfecting the engineered MHC class II+ tumor cells with the *Ii* gene abrogated the vaccine potential of the modified cells. The destruction of tumor cell immunogenicity following re-introduction of the *Ii* gene presumably resulted from its ability to block the binding of endogenous tumor-associated peptides in the endoplasmic reticulum and consequent development of a Th cell response to epitopes from those peptides. Both CD4+ Th cells and CD8+ CTLs were found to be essential, as deletion of either T-cell subpopulation in adoptive transfer of immunity experiments abrogated the protective effect [25]. This study is consistent with the established principle that the activation of CD4+ Th cells by MHC class II+/Ii- tumor cells is required for optimal activation and expansion of CD8+ CTLs. Finally, the importance of Th cells activated by MHC class II+/Ii- tumor cells in prolonging immunological memory was demonstrated by the protection of mice against tumor challenge for extended periods of time after vaccination [25].

Figure 1. Tumor malignancy and Ii expression.



When tumor cells are MHC class II+ and Ii+, the tumor is highly malignant, as Ii blocks presentation of tumor antigen through MHC class II. The same situation applies to tumor cells that are MHC class II- and Ii-. Only when they are MHC class II+ and Ii- do tumor cells present the tumor antigen by MHC class II to activate CD4+ T-cells, which in turn activate CD8+ CTLs.

Endogenous proteins from different intracellular compartments of a tumor cell can be presented by MHC class II+/Ii- tumor cells [26,27]. This is demonstrated by the finding that cells engineered to express the gene for hen egg lysozyme (HEL) with a leader sequence targeting the endoplasmic reticulum, presented MHC class II HEL epitopes to HEL-specific CD4+ T-cells. Similar to the studies described above, co-expression of the Ii protein in these cells inhibited presentation of the HEL epitopes [27].

Antisense suppression of Ii in tumors induced for MHC class II and Ii expression

Given the numerous different MHC class II alleles in humans, even only at the human leukocyte antigen (HLA)-DR locus, generating the MHC class II+/Ii- phenotype by transfecting a patient's tumor with genes matching MHC class II α and β chain types is not a practical clinical approach. We developed an alternative approach using a non-polymorphic gene construct active in all humans. Expression of endogenous MHC class II molecules can be induced using *CIITA* or *IFN*γ genes, while the co-induced Ii protein is suppressed with a reverse gene construct (*Ii*-RGC) targeting Ii mRNA. *Ii*-RGC acts at the mRNA level to prevent translation of the Ii protein. Initially, Ii antisense oligonucleotides were used to suppress Ii expression in MHC class II+/Ii+ tumor cells [28]. In these studies, using the Sal 1 sarcoma model, MHC class II+ cells treated with Ii antisense oligonucleotides demonstrated good vaccine potency against challenge by parental tumor.

Subsequently, we created expressible Ii antisense constructs (*Ii*-RGC) for inclusion into DNA vaccine vectors. These constructs were cloned into expressible plasmids and/or engineered into adenoviruses to evaluate different methods of tumor gene transfection [29,30]. The *Ii*-RGCs were evaluated by stable or transient DNA transfections using several murine tumor cell lines; the most active, *Ii*-RGC(-92,97) (A in the AUG start codon is position 1), was selected for *in vivo* studies.

While some tumor cell lines were MHC class II+/Ii+, many of the lines we tested were MHC class II-/Ii-. In these cell lines, the *CIITA* or *IFN*γ gene, or both, were co-transfected *in vitro* with *Ii*-RGC(-92,97) to create the MHC class II+/Ii- phenotype, as detected by immunostaining [29-31]. *In vivo* induction of this phenotype in established tumors was also generated by intratumoral injection of *Ii*-RGC and *CIITA* plasmids delivered in liposomes [29,31], or using recombinant adenoviral vectors containing *Ii*-RGC(-92,97), *CIITA* and *IFN*γ [30]. While the *CIITA* gene used in the mice studies is human, its gene product fortuitously functions well on the murine promoters for MHC class II and *Ii* genes [30].

Ii suppression therapy of animal tumors

We tested our therapeutic strategy for both tumor prevention (vaccination to protect against tumor challenge) and tumor cure (therapy of established tumors). In a prevention model using Sal 1, tumor cells treated with antisense oligonucleotide-suppressed Ii protein were much more potent than Sal 1 cells treated with sense and mismatch antisense oligonucleotides [28]. In cure models, the *in vivo* activities of these therapeutic constructs were tested by intratumoral injection of plasmids or adenoviral vectors in established subcutaneous tumors of both the Renca renal adenocarcinoma and RM-9 prostate carcinoma murine models [29,31].

In tumor cure models, complete regression of established tumors was achieved. Renca tumor regression was observed in approximately 50% of mice following four intratumoral injections of *CIITA* and *Ii*-RGC plasmid constructs over 4 days administered with a subtherapeutic dose of *IL-2* plasmid [29]. In these tumor nodules, *in situ* induction of the MHC class II+/Ii- phenotype was confirmed by immunohistochemical staining of tumor sections [29]. The injection of established Renca tumors with recombinant adenovirus, containing *CIITA*, *IFN*γ and *Ii*-RGC, combined with a low suboptimal dose of *IL-2* adenovector, induced complete tumor regression in approximately 60 to 70% of mice and complete protection against Renca tumor re-challenge [30]. These studies using the weakly immunogenic Renca MHC class I+/class II- model confirm that induction of the MHC class II+/Ii- phenotype triggers an antitumor immune response with long-lasting systemic immunity.

In the aggressive, poorly immunogenic MHC class I-/class II- RM-9 prostate tumor model, *in situ* induction of the MHC class I+/class II+/Ii- phenotype by intratumoral injection of the plasmids *pCIITA*, *pIFN*γ and *pIi*-RGC caused a significant but transient inhibition of tumor growth, even when suboptimal doses of pIL-2 were added to the tumor nodule treatment (Table 1) [31]. Complete responders were observed only when tumor nodules were first irradiated prior to gene therapy [31]. In a subsequent experiment, established RM-9 subcutaneous tumors were selectively irradiated and treated 1 day later with intratumoral plasmid gene therapy using a mixture of the plasmids *pCIITA*, *pIFN*γ and *pIi*-RGC combined with a subtherapeutic dose of *pIL-2* for 4 consecutive days. Table 1 demonstrates that intratumoral treatment with all four plasmids induced complete tumor regression in more than 50% of the mice only when tumor irradiation was administered 1 day prior to gene therapy. Mice rendered tumor free by radiation and intratumoral gene therapy and re-challenged on day 64 were protected against RM-9 challenge but not against syngeneic EL-4 tumor challenge (Table 1) [31]. These findings demonstrate that in the RM-9 model, radiation enhanced the therapeutic efficacy of intratumoral gene therapy for *in situ* induction of tumor-specific immunogenicity.

IL-2 at subtherapeutic doses is probably acting as an adjuvant to strengthen and sustain the activation of T-cells. Our recent studies demonstrate that both CD4+ Th cells and CD8+ CTLs are essential for the induction of a complete antitumor response in the RM-9 model, specifically, the *in vivo* depletion of either T-cell subset abrogates this response [GG Hillman *et al*, unpublished data]. These studies are consistent with induction of both a Th response and a CTL response by our gene therapy approach, resulting in long-lasting tumor immunity. Furthermore, in both the Renca and RM-9 model, omission of the *Ii*-RGC vector in the intratumoral gene therapy protocol led to a significantly lower incidence of complete tumor regressions, emphasizing the essential role of Ii suppression in the induction of a complete and systemic antitumor immune response [30,31].

Table 1. Antitumor response of RM-9 tumor-bearing mice treated with radiation and plasmid gene therapy, and response of cured animals to re-challenge.

Treatment group	Proportion of tumor-free mice		
	Post-treatment	Post-challenge	
		RM-9	EL-4
Control	0/10	-	-
pCIITA + pIFNγ+ pIL-2 + pIi-RGC	0/7	-	-
Radiation	0/11	-	-
Radiation + *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC	7/13	7/7	-
Radiation + *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC	3/6	-	0/3
Naive mice	N/A	0/5	0/5

Established RM-9 tumors of 0.3 to 0.4 cm were irradiated with 8 Gy photons on day 6 after cell injection. From day 7, tumors were injected with the plasmids *pCIITA + pIFNγ+ pIL-2 + pIi*-RGC for 4 consecutive days. The proportion of tumor-free mice is presented at the end of the observation period, on day 64, following radiation and plasmid therapy. Tumor-free mice and naive mice were re-challenged with RM-9 cells or genetically identical EL-4 cells on day 64, the proportion of tumor-free mice by day 30 after challenge is reported [31]. **N/A** not applicable.
(Reproduced with permission from Mary Ann Liebert and Hillman GG, Xu M, Wang Y, Wright JL, Lu X, Kallinteris NL, Tekyi-Mensah S, Thompson TC, Mitchell MS, Forman JD: ***Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma.*** *Human Gene Therapy (2003)* ***14****(8):763-775. © 2003 Mary Ann Liebert).*

Role of radiation

The role of radiation in enhancing intratumoral gene therapy for the induction of cancer immunity is particularly intriguing. Possible mechanisms for radiation enhancement of gene therapy include the following: (i) the DNA damaging and debulking effect slows tumor growth to allow time for the immune response to be effective [31,32]; (ii) radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity that can be readily activated by *in situ* gene therapy [32]; (iii) radiation is hypothesized to limit suppressive immunoregulatory T-cells; and (iv) radiation increases gene transduction efficiency and duration of expression of surviving tumor cells, thus improving the efficiency of *in situ* genetic modification, leading to an immune response that eradicated remaining tumor cells. Stevens and colleagues demonstrated that radiation improves the transfection efficiency of plasmid DNA in normal and malignant cells *in vitro* resulting from radiation-induced DNA breaks and DNA repair mechanisms [33,34]. They demonstrated that radiation followed by plasmid or adenoviral transfection caused enhanced integration of the transgene. Preliminary studies in the Renca model using intratumoral injections of the *IL-2* adenovector demonstrated that radiation potentiates the genetic modification of the tumor cells by increasing both the level and duration of expression of transfected genes [GG Hillman, unpublished data].

Unique features of Ii antisense therapy

Making antisense therapy effective clinically for other cancer molecular targets has two daunting obstacles. One is the requirement for delivering antisense reagents to all tumor cells, and the other is the requirement for suppressing the target gene continuously [35•,36]. Comparatively, Ii suppression for tumor immunotherapy has three advantages. Firstly, it stimulates tumor antigen-specific CD4+ Th cell activation without interrupting MHC class I presentation for CD8+ CTL activation. Simultaneous activation of both CD4+ and CD8+ T-cells creates a more robust tumor cell immunotherapy. Secondly, inhibition of Ii protein expression does not need to occur in all tumor cells, unlike antisense targeting other tumor genes. Thirdly, the Ii inhibition does not need to be continuous, as a transient inhibition of Ii protein (3 to 5 days) in a portion of tumor cells is sufficient to generate a strong antitumor immune response [30,31]. After a specific antitumor immune response has been generated, the immune system will eradicate the residual tumor cells until all tumor cells sharing the same tumor antigens have been killed.

Future directions

Issues to address in the design of clinical trials

The biological mechanism of this therapy leads to special considerations for the design and evaluation of clinical trials. For initial trials, patients with metastatic disease poorly responsive to other means of therapy will be targeted. Although chemotherapy in such patients suppresses the anticancer immune response, once leukocyte counts have rebounded, this mode of immunotherapy eliciting an immune response within injected tumor masses, is possible. Readily accessible, ie, subcutaneous axillary masses in breast carcinoma might be preferred because they can be approached with ease. However, many radiologists feel that any tumor they can visualize is a candidate for intratumor injections, for example, colon adenocarcinoma. While melanoma has been a classic target for tumor immunotherapy procedures, the frequency of accessible masses, frequency of patients with metastatic disease, and a variable and sometimes long clinical course, indicates against melanoma for initial trials.

Injection of a tumor with *Ii*-RGC is expected to lead to presentation of normal tissue antigens, as well as tumor determinates. While we have not observed histological signs of autoimmunity in mice with cancers treated by this method, such reactions against self-tissues probably occur. Those reactions on balance might promote tumor cure. Vitiligo is observed in some melanoma patients vaccinated against melanoma-associated tumor antigens. Signs of autoimmunity to normal tissue antigens of the tumor will be monitored in trials of this immunotherapy.

Additional therapeutic uses for Ii suppression

This technology can also be used to identify novel MHC class II epitopes in tumor and autoimmune disease-related antigens. Specifically, tumor- and autoimmune disease-related antigenic epitopes can be identified by high performance liquid chromatography (HPLC) tandem mass

spectrometry of acid-eluted peptides from immune purified MHC class II molecules. The eluted-peptide HPLC patterns can then be compared with those of MHC class II+/Ii+ cells to identify the putative Ii suppression of specific peaks [37•]. The molecular weight of a peptide in such a peak can be precisely determined by tandem mass spectrometry and a sequence assigned from the weight.

Ii suppression can also enhance the efficiencies of DNA vaccines and of gene-transfected DC vaccines. The biological effect of Ii suppression might enhance the immune response to a co-delivered DNA vaccine containing a gene for a malignant or infectious antigen [38-40]. When a cell (eg, a professional APC) is transfected with a gene encoding an antigen and an Ii suppression construct, this cell expresses the antigen endogenously, while Ii is suppressed to produce the MHC class II+/Ii- phenotype. Consequently, the transfected cell can now present antigenic epitopes through MHC class II and I to activate both CD4+ Th cells and CD8+ CTLs, respectively. The result is a stronger DC or DNA vaccine.

Conclusion

We have developed a novel antisense approach to convert tumor cells into MHC class II+ and Ii- APCs. Suppression of Ii gene expression in the endoplasmic reticulum leads to the simultaneous presentation of endogenous tumor antigens by both MHC class I and II molecules and generates a robust and long-lasting antitumor immune response. Injecting murine tumors with genes that induce the MHC class II+/Ii- phenotype in tumor cells causes complete tumor regression (ie, cure) in a significant number of animals with renal and prostate tumors.

Compared with other antisense applications, our Ii antisense method has two major advantages; Ii only needs to be suppressed temporarily and it does not need to be suppressed in all tumor cells. Analogous human Ii antisense gene constructs that are suitable for most patients and cancers have been developed.

Conversion of cancer cells into APCs via induction of the MHC class II+/Ii- phenotype *in vivo* by this method is simple to achieve. The induction of MHC class II molecules and Ii by *CIITA* and suppression of Ii by *Ii*-RGC antisense is a clinically practical strategy, as both *CIITA* and *Ii* genes are monomorphic. Transduction of a focal population of cells within a tumor mass stimulates an immune response that potentially leads to destruction of all tumor cells within that mass as well as in distant metastases. This is particularly relevant to the anticipated clinical use where local treatment to induce a potent systemic antitumor immune response is the goal.

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• of special interest

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LABORATORY—CLINIC INTERFACE

Turning tumor cells in situ into T-helper cell-stimulating, MHC class II tumor epitope-presenters: immuno-curing and immuno-consolidation

Gilda G. Hillman[a], Nikoletta L. Kallinteris[b], Xueqing Lu[b], Yu Wang[a], Jennifer L. Wright[a], Yu Li[b], Shuzhen Wu[b], Jeffrey D. Forman[a], Joseph V. Gulfo[b], Robert E. Humphreys[b], Minzhen Xu[b,*]

[a] *Department of Radiation Oncology, Barbara Ann Karmanos Cancer Institute at Wayne State University School of Medicine, 4100 John R., Detroit, MI 48201, USA*
[b] *Antigen Express Inc., 100 Barber Avenue, Worcester, MA 01606-2478, USA*

KEYWORDS
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T helper cells

Summary Immunological control or cure of tumors depends on initiating a robust T helper cell response to MHC class II epitopes of tumor-associated antigens. T helper cells regulate the potency of cytotoxic T lymphocyte and antibody responses. We have developed a novel approach to stimulate T helper cells by converting tumor cells into MHC class II molecule-positive, antigen presenting cells. Furthermore, using antisense methods, we suppress expression of the Ii protein, that normally blocks the antigenic peptide binding site of MHC class II molecules during synthesis in the endoplasmic reticulum. In such gene-engineered tumor cells, the MHC class II molecules pick up antigenic peptides, which have been transported into the endoplasmic reticulum for binding to MHC class I molecules. All nucleated cells create such "surveys of self" to detect viral or malignant transformation. Our method extends that survey of self to MHC class II endogenous tumor-associated antigens. Simultaneous presentation of tumor antigens by both MHC class I and II generates a robust and long-lasting antitumor immune response. Injecting murine tumors with genes, which induce MHC class II molecules and suppress Ii protein, cures a significant number of animals with renal and prostate tumors. We have developed analogous human gene vectors that are suitable for most patients and cancers, because they are monomorphic and active in all HLA-DR alleles. We review our findings, and analyze remaining issues for preclinical study and the design of clinical trials.

Introduction

By inducing major histocompatibility complex (MHC) class II molecules on tumor cells in situ and suppressing the immunoregulatory Ii invariant

*Corresponding author. Tel.: +1-508-852-8783; fax: +1-508-852-8653.
E-mail address: minzhenxu51@yahoo.com (M. Xu).

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chain protein, we have developed a potent tumor cell autovaccine therapy for many tumors.[1–4] This method uses simple gene-regulating reagents potentially usable in all patients, regardless of histotype.

Most tumors are MHC class II molecule-negative and cannot directly stimulate CD4+ T helper cells, which otherwise would up-regulate cytotoxic T lymphocyte (CTL) and antibody responses against the tumor. We induce MHC class II molecules in the tumor cells by transfecting genes for MHC class II transactivator (CIITA) or interferon-gamma (IFN-γ). However, we must then suppress the co-induced immunoregulatory protein Ii by antisense methods. The normal function of the Ii protein is to block the antigenic peptide binding site of MHC class II molecules at synthesis in the endoplasmic reticulum (ER), until the proteolytic release of Ii occurs in a post-Golgi antigenic peptide charging compartment. By suppressing the Ii protein in the ER, nascent MHC class II molecules bind peptides transported there for binding to MHC class I molecules. In such cells, tumor peptides, which have been processed and transported into the ER, are bound to and presented by both "unblocked" MHC class II molecules and MHC class I molecules (Fig. 1).

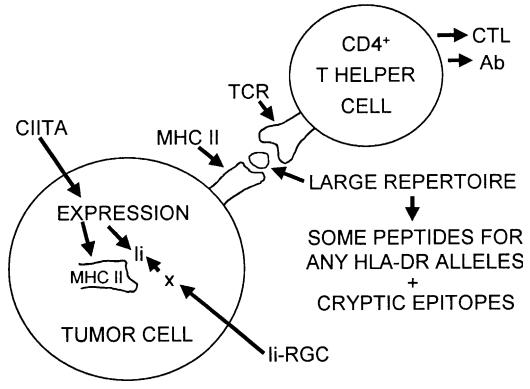

Figure 1 Summary of mechanism. Transduction of genes for either MHC class II transactivator (CIITA) or interferon-γ induces tumor cells to express MHC class II molecules and the antigenic peptide binding site binding protein Ii. Co-transduction of a reverse gene construct for a segment of Ii induces an antisense mRNA (X) which blocks transcription of Ii protein. Without the Ii protein, MHC class II molecules in the ER bind a large repertoire of endogenous immunogenic peptides including cryptic epitopes. In Ii-suppressed tumor cells, MHC class I presentation is not interrupted by Ii suppression and thus such tumor cells present simultaneously through both MHC class I (not shown in the figure) and class II molecules to CD8+ and CD4+ T cells, respectively. Such simultaneous presentation greatly enhances the activation of CTL. Among the large repertoire of MHC class II-presented peptides are additional ones to be presented by many MHC class II alleles, plus novel epitopes which were previously not dominantly presented, and are therefore candidates for breaking immunosuppression to the cancer.

Induction of such MHC class II-positive, Ii-suppressed tumor cells leads to presentation of a large repertoire of T helper cell-recognized epitopes, with no need to identify each patient's MHC class II histotypes. In mice, this therapy induces both T helper and CTL responses, curing established tumors. This autovaccine therapy protects against subsequent challenge with the same tumor but not another unrelated syngeneic tumor. Our genetically controlled immunotherapy needs to transform only a fraction of the cells in a treated nodule, in order to establish a potent, systemic immune response capable of eradicating non-transduced tumor cells.

Intratumoral gene transfections, with adenovirus vectors or DNA plasmid vectors delivered in liposomes, work well in murine models of renal cell and prostate adenocarcinomas. Human reagents are being developed for clinical trials. This immunotherapy is augmented with low levels of IL-2, IFN-γ cytokine genes and radiation. We present here the mechanism, clinical potential, and a roadmap to clinical trials for this novel approach to control or cure many human cancers.

Relevant basic immunology

The immune system uses T lymphocytes to identify and control malignant or viral transformation in all cells of the body. CTL recognizing non-self, peptide epitopes expressed on MHC class I molecules, can kill the transformed cell. The surveillance of self-peptides originates with proteosome digestion of cytoplasmic proteins into peptides, which are transported into the ER by the transporter of antigenic peptides (TAP). That repertoire of self-peptides become bound to MHC class I molecules in the ER, at the time of their synthesis, and transported to the cell surface for recognition by CD8+ CTL. MHC class I molecules are expressed on all nucleated cells of the body.

CD4+ immunoregulatory T helper cells recognize antigenic peptides presented by MHC class II molecules on professional antigen presenting cells (APC), e.g., dendritic cells (DC), macrophages, and B lymphocytes. In such APC, MHC class II molecules do not normally bind the ambient peptides of the ER at the time of their synthesis, because the antigenic peptide binding site of MHC class II molecules is blocked by the Ii protein. The trimer consisting of Ii protein, and MHC class II alpha and beta chains is transported to a post-Golgi antigenic peptide charging compartment where proteases,

which also digest internalized antigenic proteins, cleave and release Ii protein in a concerted process of Ii fragments release/antigenic peptide charging.[5–7] Normally, only exogenous antigens, that are selected for internalization by the APC, are processed for MHC class II presentation. In the case of DC, recognition of MHC class II epitopes activates CD4+ T helper cells. Activated CD4+ T helper cells, in response to that epitope, leads to maturation (licensing) of the DC to stimulate CTL recognizing MHC class I-presented peptides.[8] Likewise, B lymphocytes recognizing MHC class II epitopes processed from antigens internalized by B cell surface immunoglobulins, activate the B lymphocytes to proliferate and mature into antibody secreting plasma cells.

Suppressing expression of the Ii protein by antisense methods leads to MHC class II molecules picking up peptides from the repertoire transported into the ER (Figure 1). Such tumor cells then present tumor antigens to both CD4+ and CD8+ T cells. Activated $CD4^+$ T cells, which are specific for endogenous tumor antigens, contribute to in situ licensing of tumor cells (tumor cell APC). The licensing process involves the in vivo induction of B7 through MHC class II molecules.[9] Transfection of tumor cells with cytoplasmic region deleted MHC class II lost the capability to induce the expression of B7 in vivo.[9] CIITA is a master transcription factor that induces the expression of MHC class II molecules in all tumor lines we have tested.[1–4] Sal 1 sarcoma line,[1] MC-38 colon adenocarcinoma[3] and Renca renal adenocarcinoma[3] retained good responses to IFN-γ, with MHC class II molecules being induced to same extent as to that induced by CIITA. However, in RM-9 prostate carcinoma cells CIITA must be used to induce the expression of MHC class II molecules.[4] RM-9 cells may be defective in the CIITA gene and IFN-γ cannot induce MHC class II molecules. Nevertheless, in our studies, IFN-γ is required for optimal protection in the RM-9 murine prostate carcinoma model in addition to CIITA to induce MHC Class II molecules.[4] IFN-γ induces MHC class I molecules on the transfected RM-9 tumor cells, consistent with the hypothesis that tumor cells are converted into APC-surrogates to activate CTL.

Pioneer work exploiting use of MHC class II-positive/Ii-negative phenotype in tumor immunotherapy

The concepts underlying our work were identified first by Dr. Suzanne Ostrand-Rosenberg and colleagues. They demonstrated that transfecting syngeneic genes for MHC class II alpha and beta chains into a MHC class II-negative tumor, creates a tumor cell vaccine, which protects against challenge with the parental tumor.[10–12] In the murine Sal sarcoma model, the parental tumor is MHC class I-positive, but MHC class II-negative. In mice vaccinated with the gene-engineered MHC class II-positive cells, both CD4+ T helper cells and CD8+ CTLs were essential for protection against challenge by parental cells, because antibody-mediated deletion of either cell population, destroyed the protective response.

Supra-transfecting the potent, engineered MHC class II-positive tumor cells with a gene for the Ii-protein, abrogated the vaccine potential of the modified cells.[11] That is, the engineered cells were no more potent as vaccine cells than were the parental cells. In the potent vaccine MHC Class II+/Ii-suppressed cells, MHC class II molecules, not blocked by the Ii protein at the time of their synthesis, picked up ambient peptides (including tumor peptides) in the ER. Introducing expression of the Ii protein into such cells again, blocked binding of the ER peptides and destroyed the immunogenicity of the tumor cells, even if MHC class I presentation continued. In these experiments, the T helper cells activate and expand the population of CD8+ CTL. This enhancement of the CTL response seems to be mediated by the "licensing" activity of CD4+ T cells on tumor cells to become APC here, which in turn activate CD8+ T cells more potently.[10] An additional function of T helper cells, activated by MHC class II-positive/Ii-suppressed tumor cells, has been to prolong memory and protecting mice against tumor challenge for long periods of time after vaccination.[11]

This group further demonstrated that endogenous proteins from many intracellular compartments of a tumor cell can become presented by the MHC class II-positive/Ii-suppressed tumor cells.[13–15] The gene for hen egg lysozyme (HEL) was engineered with leader sequences targeting to the ER. MHC class II epitopes of HEL were presented to HEL-specific CD4+ T cells when transfected into cells, which were MHC class II-positive but Ii-negative. Co-expression of Ii protein in such cells inhibited presentation of the HEL epitopes. Absence of H-2M, another regulator of antigenic peptide charging to MHC class II molecules, had no effect on endogenous tumor antigen presentation in this model.[14]

Clinically practical tools and methods

In a clinical setting, it is not feasible to transfect a patient's cells with autologous MHC class II genes

because MHC class II alleles are highly polymorphic. We have used genetic tools to induce endogenous MHC class II molecules (with CIITA or IFN-γ) and suppress Ii protein (by antisense methods, oligonucleotides or reverse gene constructs).[1–4] Tumor cells treated with an active Ii antisense oligonucleotide were potent vaccine cells.[1] However, since antisense oligonucleotides have limited use in vivo, we created expressible Ii antisense gene constructs. Since there is only one human Ii allele, our reagents are therefore suitable for use in all patients regardless of MHC class II allele polymorphism.

Design and in vitro testing of gene constructs

We first synthesized Ii antisense oligonucleotides to suppress Ii expression in MHC class II+/Ii+ tumor cells.[1] In the sarcoma cell (Sal1) tumor model, tumor cells treated with this Ii antisense oligonucleotide are potent vaccine against challenge by parental tumor. In order to develop clinically useful in vivo therapeutic antisense reagents, we also created expressible Ii antisense reverse gene constructs (Ii-RGC). These were constructed by cloning different Ii gene fragments in reverse orientation into expressible plasmids or adenoviruses, to evaluate multiple methods of tumor cell administration.[3,4] The Ii-RGC genes were evaluated by stable or transient DNA transfections in several murine tumor cell lines, including A20 lymphoma cells, MC-38 colon adenocarcinoma cells, Renca renal adenocarcinoma cells, B16 melanoma cells, and RM-9 prostate cancer cells. The most active one Ii-RGC (−92,97) (A in the AUG start codon is position 1) was chosen for in vivo studies.

Among the cell lines tested, A20 is already MHC class II+/Ii+. Ii-RGC (−92,97) significantly inhibited Ii expression when this construct was delivered by lipid or gene gun transfection methods. The other tumor lines tested are MHC class II−/Ii−. These cell lines were co-transfected in vitro with Ii-RGC (−92,97) and either CIITA or IFN-γ, or both, creating the MHC class II-positive/Ii-suppressed phenotype.[2–4] *In vivo* induction of the MHC class II-positive/Ii-suppressed phenotype was also generated by intratumoral injection of Ii-RGC and CIITA plasmids with lipid[2,4] or recombinant adenoviral vectors containing Ii-RGC (−92,97), CIITA and IFN-γ.[3] In summary, we have generated therapeutic MHC class II-positive/Ii-suppressed phenotype induction constructs, which are biologically very active in all tested cell lines.

Efficacy in "tumor cure" models

The in vivo activities of these therapeutic constructs were tested by intratumoral injection in established subcutaneous tumors using two tumor models: the Renca renal carcinoma and the RM-9 prostate carcinoma. In both tumor models, complete regression of established tumors was achieved. In the Renca model, tumor regression was observed in about 50% of mice following four intratumoral injections of CIITA and Ii-RGC plasmid constructs over four days given together with a suboptimal dose of IL-2 plasmid.[2] Intratumoral injections of recombinant adenovirus, containing CIITA, IFN-γ, Ii-RGC constructs and IL-2 gene, in established Renca tumors induced complete tumor regression in about 60—70% of mice and protection against Renca tumor rechallenge.[3] In an aggressive, poorly immunogenic RM-9 prostate tumor model, radiation augmented the effect of the suboptimal dose of IL-2 and MHC class II-positive/Ii-suppressed phenotype causing complete tumor regression in 50% of the mice.[4] Established RM-9 subcutaneous tumors were selectively irradiated and treated a day later with intratumoral plasmid gene therapy using the plasmids pCIITA, pIFN-γ, pIL-2 and pIi-RGC for four consecutive days. Data presented in Table 1 showed that intratumoral treatment with all the four plasmids induced complete tumor regression in more than 50% of the mice only when tumor irradiation was administered one day prior to gene therapy. Mice rendered tumor-free by radiation and intratumoral gene therapy and re-challenged on day 64, were protected against RM-9 challenge but not against syngeneic EL-4 challenge (Table 1). These findings demonstrate that in the RM-9 model, radiation enhanced the therapeutic efficacy of intratumoral gene therapy for in situ induction of tumor-specific immune response.

Established RM-9 tumors of 0.3—0.4 cm were irradiated with 8 Gy photons on day 6 after cell injection. From day 7, tumors were injected with the plasmids pCIITA + pIFN-γ + pIL-2 + pIi-RGC for four consecutive days. The proportion of tumor-free mice at the end of the observation period, on day 64, following radiation and plasmid therapy, is presented. Tumor-free mice and naïve mice were re-challenged with RM-9 cells or unrelated EL-4 cells on day 64, the proportion of challenge-tumor free mice by day 30 is reported.

Reproduced from Hillman et al., Human Gene Therapy 2003; 14: 763—775.[4]

In order to obtain optimal therapeutic effect, MHC class II and Ii must be induced with CIITA and Ii needs to be inhibited by Ii-RGC in both the Renca

Table 1 Anti-tumor response of RM-9 tumor-bearing mice treated with radiation and plasmid gene therapy, and response of cured animals to re-challenge

	Tumor-free mice		
	Post treatment	Post challenge tumor	
Treatment group		RM-9	EL-4
Control	0/10	—	—
PCIITA + pIFN-γ + pIL-2 + pIi-RGC	0/7	—	—
Radiation	0/11	—	—
Radiation + empty plasmid	0/5	—	—
Radiation + pCIITA + pIFN-γ + pIL-2 + pIi-RGC	7/13	7/7	—
Radiation + pCIITA + pIFN-γ + pIL-2 + pIi-RGC	3/6	—	0/3
Naïve mice	NA	0/5	0/5

and RM-9 tumor models.[2–4] Our results are consistent with those of Martin et al.[16] who showed, in a murine lung carcinoma model, that induction of MHC class II by CIITA did not create an efficient tumor cell vaccine. This study confirms our finding that induction of MHC class II by transfecting CIITA, which also induces Ii, is insufficient for a therapeutic effect. One must obtain the therapeutic phenotype of MHC class II+/Ii− by also suppressing Ii protein. In order to test for optimal suppression of Ii protein, our therapeutic constructs CIITA and Ii-RGC were used at different ratios. At least a 1:4 ratio (CIITA:Ii-RGC) was required to ensure good inhibition of Ii. IFN-γ is used in the RM-9 prostate tumor to induce MHC class I molecules which are not expressed in the parental cells. Renca cells are MHC class I-positive cells and IFN-γ is not needed to induce MHC class I molecules but does up-regulate further their expression. In both tumor models, a subtherapeutic dose of IL-2 plasmid is needed to promote the immune response.

Given this clear demonstration of efficacy in curing established tumors in mice, and steady progression in preclinical studies to determine optimal treatment protocols, we have begun to develop reagents for treating human cancers. The CIITA gene we used in the mice studies is human and its product functions well on the murine promoters for MHC class II and Ii genes.[17] We also made several human Ii-RGCs, which inhibited Ii expression in a human B lymphoblastoid and the HeLa cell lines. Figure 2 presents the human Ii-RGC (hIi-RGC) induced inhibition of Ii expression in HeLa cells. Transduction of cells with CIITA construct induced up-regulation of cell surface MHC class II molecules and intracellular Ii while transduction of cells with both CIITA and hIi-RGC caused suppression of Ii without affecting enhanced expression of MHC class II. These data were reproduced in additional human tumor cell lines including the human B lymphoma cell line Raji, and human melanoma cell line. We now have in hand the Ii-RGC reagents needed for clinical trials, including human IFN-γ in an expression plasmid.

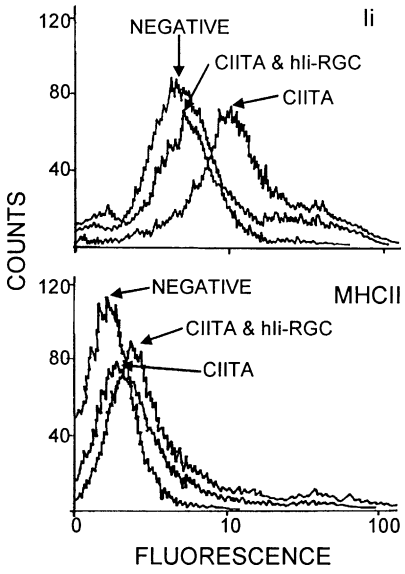

Figure 2 Reagents for treatment of human tumors. Human cervical carcinoma HeLa cells were transduced with MHC class II transactivator (CIITA) or both CIITA and Ii reverse gene construct (hIi-RGC). The cells were immuno-stained for cell surface MHC class II molecules (MHC II) or intracellular Ii protein (Ii) and analyzed by flow cytometry. The human Ii-RGC suppressed Ii protein without affecting MHC class II expression.

Continuing questions in preclinical development

What roles do cytokines play?

In all intratumoral studies a low dose of IL-2 was needed for optimal therapeutic effect. Induction of MHC class II-positive/Ii-suppressed phenotype by treatment with CIITA and Ii-RGC constructs only without IL-2 construct was not sufficient to induce a complete tumor regression, consistent with the observation that injecting only CIITA and Ii-RGC vectors does not elicit an appreciable T cell infiltration into the tumor site.[2] Addition of an IL-2 gene plasmid provided for local release of IL-2 to promote T cell infiltration and activation.[18] Intratumoral IL-2 plasmid therapy alone, at higher doses

of 50 μg/injection, in Renca tumors and ovarian tumors was tumoricidal.[18,19] However, in our studies, using a subtherapeutic low dose of 2 μg/injection of IL-2 plasmid together with in vivo induction of the MHC class II-positive/Ii-suppressed phenotype effectively shrank or greatly reduced the rate of progression of established Renca tumors.[2] These findings were confirmed in our Renca studies using the same approach with adenoviral vectors.[3] The addition of the IL-2 gene enhanced the response probably by acting as an adjuvant cytokine, helping to strengthen and sustain the activation of both CD4+ and CD8+ T cells. Our data indicate that CIITA plus Ii-RGC alone displays endogenous tumor antigen by both MHC class I and II to initiate antitumor immune response. Induction of a full antitumor immune response requires promotion of T cell proliferation. In RM-9 cells that are MHC class I-negative, addition of IFN-γ gene to CIITA and Ii-RGC genes was required for optimal therapeutic effect.[4] IFN-γ induces expression of MHC class I molecules and also enhances the maturation of DC and NK cells. Our early study showed that IFN-γ plus Ii antisense oligonucleotide inhibition in Sal1 cells offered a more potent antitumor protection than CIITA plus Ii antisense oligonucleotide.[1] IFN-γ is not required to induce a potent therapeutic effect in Renca model since Renca cells are MHC class I-positive.[20] Whether MHC class II-positive/Ii-suppressed phenotype immunotherapy might be used with other cytokines is an open question. Theoretically, CIITA plus Ii-RGC should synergize with other methods to enhance the immune response, such as B7 gene injection.

How does radiation enhance Ii suppression immunotherapy in the prostate tumor model?

In the RM-9 prostate tumor model, a single dose of 8 Gy photon radiation, selectively administered to the tumor prior to gene therapy, enhanced the therapeutic effect of the MHC class II-positive/Ii-suppressed phenotype.[4] Complete tumor regression associated with systemic tumor immunity occurred only when established tumors were first irradiated followed a day later by initiation of intratumoral CIITA, IFN-γ, Ii-RGC and IL-2 plasmid therapy for four consecutive days. Possible mechanisms for radiation enhancement of gene therapy include the following: (1) The debulking effect slows tumor growth so that immunotherapy has time to develop. (2) Radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity. (3) Radiation limits suppressive immunoregulatory T cells. (4) Radiation increases gene transduction efficiency and duration of expression of surviving tumor cells thus increases efficiency of in situ gene modification leading to immune response.[21]

What additional preclinical studies are needed?

Additional pre-clinical studies before initiating a clinical trial include the following: (1) Toxicology and pharmacokinetics of the DNA plasmids or adenoviral vectors will be evaluated, including biodistribution and existing duration of the therapeutic vectors in different tissues and organs. (2) Therapeutic constructs will be optimized, for example by constructing a plasmid that contains both CIITA and multiple copies of Ii-RGC in order to increase the efficiency of inducing the MHC class II-positive/Ii-suppressed phenotype and to decrease the amount of the DNA vectors. (3) The injection schedule/doses of cytokines and frequency of radiation will be optimized. The ratio of injected DNA versus tumor volume needs to be determined. All of these studies are underway. A final concern is to monitor for possible induction of autoimmunity, which might be of therapeutic benefit when restricted to the tumor tissue.

How does this method compare to other in situ therapies?

Several effective therapies have been developed for in situ, as opposed to systemic, treatment of tumors. Such modalities including cryotherapy, external beam radiation, radiation seed implantation (brachytherapy), and photodynamic therapy (porfimer sodium), are approved for patients with prostate cancer, head and neck cancer, and lung cancer. Local injection of approved systemic chemotherapeutic agents is being investigated,[22,23] in addition to the intratumoral injection of the following experimental products: oncolytic viruses,[24] suicide genes,[25,26] tumor-suppressor,[27,28] and cytokine genes,[29–33] genes for immunomodulating molecules including B7;[34] and DC.[35,36]

Most of these approaches kill the affected tumor cells, but do not eradicate distant tumors. The most promising approaches are those that are designed not only to kill the cells that are directly contacted by the intratumoral therapy, but also, that elicit an immune response which in turn eradicates tumor cells and deposits at both locoregional and distant sites.[37] Intratumoral injections of cytokines such as IL-2, TNF-α, also offered substantial therapeutic

effects and elicited tumor-specific immune response. However, turning live tumor cells in situ into CD4+ and CD8+ T cell-stimulating cells is novel and a potent method to induce the widest spectrum of tumor antigen-specific immune responses, and more importantly, this method is synergistic with most other methods mentioned above for an improved therapeutic effect.

Is there an autoimmune response against normal tissues?

Most tumor immunotherapies or vaccines, such as tumor cell-based tumor vaccine and heat shock protein tumor vaccine, face a recurring question. Is there an autoimmune response being induced against normal tissues? In the course of the response to our immunotherapy, as well as other immunotherapies, some functionally important, self-antigens might be exposed to the immune system. We have not had evidence for autoimmune responses in immunopathological studies of 15 organs from surviving mice receiving either antisense oligonucleotides or Ii-RGC treatment (unpublished observation). Furthermore, Ii knockout mice did not have evidence of autoimmune disease.[38] Two possible explanations might account for this phenomenon: (1) Tumor antigens are usually abundant or mutated and thus are of much stronger immunogenicity while normal antigens are tolerated during development and are of much weaker immunogenicity. (2) Autoimmunity induction is organ- or tissue-specific. In the Sal1 sarcoma model, no autoimmunity was induced.[1] However, this finding does not mean that no autoimmunity is induced in other tumor model. For example, in Hashimoto's thyroiditis, we have observed discordant expression of MHC class II and Ii, suggesting MHC class II+/Ii− thyrocytes may present endogenous antigens to induce thyroiditis.[39] Finally, one must consider the possibility that a local autoimmune response within the injected tumor probably contributes to the tumoricidal effect.

Are there other uses of MHC class II-positive/Ii-suppressed phenotype-inducing constructs?

Other potential uses of MHC class II-positive/Ii-suppressed phenotype induction include defining tumor antigens and enhancing DNA vaccines. Tumor-related antigenic epitopes can be identified by tandem HPLC mass spectrometry of acid-eluted peptides from immunopurified MHC class II molecules. Eluted-peptide HPLC patterns can be compared with those of MHC class II-positive/Ii-positive cells to identify putative Ii suppression-specific peaks. The molecular weight of a peptide in such a peak can be precisely determined by tandem mass spectrometry and a sequence imputed from the weight. An alternative method to identify tumor antigens, which are preferentially presented in mice with Ii-suppression treated tumors, is to develop T cell lines. Those lines can be used as indicators for those antigens in further studies with progressive fractions of tumor cell lysates. By progressive fractionation of stimulating fractions, often a specific tumor antigen gene is identified.

A second use is to enhance a DNA vaccine. The biological effect of Ii suppression will enhance the immune response to a co-delivered DNA vaccine for a malignant or infectious antigen. When administered into the skin by gene gun impelling of DNAs adsorbed to gold particles, a strong response is registered.

Clinical trials

Clinical trials of our intratumoral gene therapy will evaluate safety and efficacy. Phase I/II and II studies can be carried out in patients with accessible cancers (e.g., breast, colorectal and prostate cancers, or melanoma), however, Phase III trials will be restricted to a selected type of cancer. Intratumoral gene therapy to induce MHC class II molecules and suppress Ii protein is administered in multiple injections (e.g., once a day for 2—4 days) into one or more tumor nodules in a patient. Safety is the first issue. We will examine and test for local toxicity (pain, inflammation, and other locoregional site reactions) and systemic toxicity (hematological, hepatic or renal toxicity) and autoimmunity. Pharmacokinetics (blood and urine) will be analyzed to evaluate the biodistribution of therapeutic plasmids. The antitumor immune response will be evaluated by tumor-specific ELISPOT assays. *In vivo* induction of the therapeutic phenotype and CD4+ and CD8+ lymphocyte infiltration will be measured in early biopsies.

A focus of Phase II trials is evaluating regression in patients with refractory tumors, at both injected and distant sites. The ultimate planned optimal use of our active immunotherapy provides for consolidation therapy. However, before undertaking trials in patients induced to no evidence of disease (NED) status (usually by surgery and adjuvant chemotherapy, but with suspected micrometastatic disease), efficacy of our protocol must be demonstrated in

measurable lesions. There are two complicating issues. First, patients in Phase II trials with refractory disease will concurrently be undergoing chemotherapy, which suppresses the immune response. Secondly, measurable smaller lesions need to be present since active immunotherapy does not work well on large masses. Myelosuppression secondary to adjuvant chemotherapy can be assessed by PBMC and BM analyses.

Additional studies will also be performed in newly diagnosed patients with no clinical evidence of disease outside the resection margins. This is an ideal setting for active immunotherapy; however, much longer-term follow-up of patients is required to determine therapeutic benefit because time to progression and survival are greatly enhanced in this group of patients compared to those undergoing surgery and adjuvant chemotherapy for advanced disease.

A first clinical trial might well target colorectal adenocarcinoma for the following reasons: (1) Tumor masses are readily accessible with minimally invasive techniques including sigmoidoscopy and colonoscopy. (2) Surgery or radiation therapy + combination chemotherapy is routinely administered. (3) Accessible recurrent tumors requiring resection and second-line chemotherapy often occur. (4) Evaluation of patients for recurrence and progression are routine.

Conclusions

One advantage of intratumoral induction of the MHC class II-positive/Ii-suppressed phenotype is that prior identification of tumor antigens is not necessary. Ii "unblocked" MHC class II molecules survey the antigenic peptide pool in ER and present whatever tumor epitopes are bound in the ER to activated $CD4^+$ T cells. This provides a better chance to prevent the tumor from escaping the host's immune surveillance since a broader spectrum of heterogeneous tumor antigens is expected to be surveyed by MHC class II molecules. CIITA also enhances MHC class I expression, especially when class I expression is diminished[40] and thus class I antigen presentation is also promoted. This effect is significant because deletion of MHC class I alleles is frequently a way for tumors to escape immunosurveillance. Another advantage of MHC class II-positive/Ii-suppressed phenotype induction is that it can be synergistic with other antitumor therapies such as injections with IL-12 and B7 genes. A final advantage is that the monomorphic structure of the Ii gene means that one vector construct can be used in all patients, regardless of the heterogeneity of MHC class II alleles.

Conversion of cancer cells into APC via induction of the MHC class II-positive/Ii-suppressed phenotype in vivo by this method is simple to achieve. Transduction of a focal population of cells within a tumor mass stimulates an immune response that potentially leads to destruction of all cells within the mass as well as in metastases. This is particularly relevant to the anticipated clinical use where local treatment to induce a potent systemic antitumor response is the goal.

Intratumoral Ii-RGC therapy offers great potential in the treatment of patients with solid malignancies. The goal of therapy is "immunoconsolidation", that is, to induce a potent antitumor immune response capable of eradicating tumor cells throughout the body that are left behind following surgery or radiation, or are not killed by adjuvant chemotherapy. The concept is presented in Table 2: (1) some cells within a tumor mass are converted to APC that elicit a robust CD4+ and CD8+ T cell immune response; (2) the effector cells recognize cancer cells within the primary tumor that were not transduced as well as cells near the primary tumor and at distant sites; (3) upon recognition by the immune system, the residual cancer cells are destroyed. The development and progression of cancer is associated with the inability of the immune system to recognize rogue cells as aberrant and to attack and kill the cells.[41–43] Therefore, uncloaking the tumor cells via transduction of the MHC class II-positive/Ii-suppressed phenotype, thereby enabling induction of a potent CD4+ and CD8+ T cell antitumor immune

Table 2 Main points

- Intratumoral gene therapy to induce an antitumor immune response is feasible.
- Novel patient-specific vaccine cells are created *in vivo* with a therapeutic phenotype of MHC class II-positive/Ii-suppressed.
- A small number of transduced cells elicit a robust CD4+ T helper cell-directed, immune response against all cancer cells — locally and at distant sites. These responses lead to enhanced progression-free and overall survival.
- Active immunotherapy works best in small volume disease states.
- Active immunotherapy is a valuable adjunct to surgery and radiation for immuno-consolidation.

response, should greatly improve clinical outcomes in patients with cancer.

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Curative Antitumor Immune Response Is Optimal with Tumor Irradiation Followed by Genetic Induction of Major Histocompatibility Complex Class I and Class II Molecules and Suppression of Ii Protein

YU WANG,[1] MINZHEN XU,[2] MINGXIN CHE,[3] ERIC VON HOFE,[2] ASAD ABBAS,[1] NIKOLETTA L. KALLINTERIS,[2] XUEQING LU,[2] ZACHARY J. LISS,[1] JEFFREY D. FORMAN,[1] and GILDA G. HILLMAN[1]

ABSTRACT

Transfecting genes into tumors, to upregulate major histocompatibility complex (MHC) class I and class II molecules and inhibit MHC class II associated invariant chain (Ii), induces a potent anti-tumor immune response when preceded by tumor irradiation, in murine RM-9 prostate carcinoma. The transfected genes are cDNA plasmids for interferon-γ (pIFN-γ), MHC class II transactivator (pCIITA), an Ii reverse gene construct (pIi-RGC), and a subtherapeutic dose of adjuvant IL-2 (pIL-2). Responding mice rejected challenge with parental tumor and demonstrated tumor-specific cytotoxic T lymphocytes (CTLs). We have extended our investigation to determine the relative roles of each one of the four plasmids pIFN-γ, pCIITA, pIi-RGC, and pIL-2 in conjunction with radiation for the induction of a curative immune response. Upregulation of MHC class I with pIFN-γ or class II with pCIITA, separately, does not lead to a complete response even if supplemented with pIL-2 or pIi-RGC. An optimal and specific antitumor response is achieved in more than 50% of the mice when, after tumor irradiation, tumor cells are converted *in situ* to a MHC class I+/class II+/Ii- phenotype with pIFN-γ, pCIITA, pIi-RGC, and pIL-2. We demonstrate further that both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells are essential for induction of an antitumor response because *in vivo* depletion of either subset abrogates the response. The radiation contributes to the gene therapy by causing tumor debulking and increasing the permeability of tumors to infiltration of inflammatory cells.

OVERVIEW SUMMARY

We showed that genetic modification of murine RM-9 prostate tumor cells, *in situ*, to express major histocompatibility complex (MHC) class I and class II molecules and suppress MHC class II associated invariant chain Ii, converts those cells into a cancer vaccine. Gene therapy was delivered intratumorally using plasmids coding for interferon (IFN)-γ, CIITA, and an Ii reverse gene construct (Ii-RGC), and a subtherapeutic adjuvant dose of interleukin (IL)-2 plasmid. Complete tumor regressions, associated with the induction of a specific antitumor immune response, were obtained only when gene therapy was preceded by tumor irradiation. We now demonstrate that each of the four plasmids IFN-γ, CIITA, Ii-RGC, and IL-2, combined with tumor irradiation, are required for optimal antitumor activity. This approach causes the induction of a strong antitumor immune response, in which $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells play an essential role. Radiation enhances gene therapy by causing tumor debulking and permeability.

INTRODUCTION

SEVERAL METHODS to induce an immune response against prostate cancer, including cytokines or peptides delivered

Departments of [1]Radiation Oncology and [3]Pathology, Barbara Ann Karmanos Cancer Institute at Wayne State University School of Medicine and Harper Hospital, Detroit, MI 48201.
[2]Antigen Express, Inc., A Subsidiary of Generex Biotechnology, Corp., Worcester, MA 01606.

via expression constructs, dendritic cells or *ex vivo* vaccination with cytokine gene-modified cells, induced an immune response but with only limited clinical results (Hillman *et al*., 1999; Simons *et al*., 1999; Steiner and Gingrich, 2000; Belldegrun *et al*., 2001; Harrington *et al*., 2001; Trudel *et al*., 2003). Several clinical trials based on immunotherapy, cancer vaccines, or gene therapy to induce an antitumor immune response did not cure advanced metastatic and bulky disease, but might be effective when combined with surgery, chemotherapy, or radiation to decrease the tumor burden (Teh *et al*., 2001). While radiation using megavoltage photons (x rays) is conventional therapy for localized prostate carcinoma, residual disease resulting in disease progression occurs in a significant number of patients (Powell *et al*., 1997; Gray *et al*., 2001). A high percentage (40–50%) of patients with newly diagnosed prostate cancer have intermediate- to high-risk localized prostate cancer and are at high risk of recurrence after radiotherapy, probably as a result of residual radioresistant tumor cells and occult micrometastases (Forman *et al*., 1998; Gray *et al*., 2001). Combining radiation with an effective cancer vaccine has the potential to eradicate tumor deposits and micrometastases, both locally and at distant sites. We have developed a novel therapeutic approach for the treatment of locally advanced prostate cancer that consists of administering local tumor irradiation with the genetic induction of cancer vaccine in tumor nodules, *in situ*, using the murine RM-9 prostate carcinoma preclinical model (Hillman *et al*., 2003b).

To create a cancer vaccine that triggers a specific and systemic antitumor immune response, tumor-associated antigens (TAA) on tumor cells must be presented to helper T cells and cytotoxic T cells in the context of major histocompatibility complex (MHC) molecules via antigen presenting cells (APC) (Hillman *et al*., 2004a). We have designed a strategy to convert RM-9 murine prostate carcinoma cells *in vivo* into APCs by simultaneously upregulating MHC molecules and suppressing the invariant chain (Ii). At the time of their synthesis in the endoplasmic reticulum (ER), unlike MHC class I molecules, MHC class II molecules cannot bind endogenous antigenic peptides (Xu *et al*., 2004.). The MHC class II molecule binding site initially is blocked by Ii, a membrane glycoprotein that acts as a transport-chaperone and inhibitor of binding of endogenous antigens to newly synthesized MHC class II molecules (Koch *et al*., 1982; Stockinger *et al*., 1989; Guagliardi *et al*., 1990). This mechanism allows only exogenous peptide binding to MHC class II molecules and limits the endogenous repertoire of peptides presented by MHC Class II molecules (Clements *et al*., 1992; Qi *et al*., 2000; Hillman *et al*., 2004a; Xu *et al*., 2004.). Inhibition or absence of Ii protein increases presentation of endogenous tumor peptides by class II molecules to helper T cells, the activation of which is essential for induction of antitumor immunity (Xu *et al*., 2000, 2004; Hillman *et al*., 2004a). These concepts are based on pioneering work by Ostrand-Rosenberg and colleagues demonstrating that transfecting syngeneic genes for MHC class II α and β chains into a MHC class II-negative tumor creates a tumor cell vaccine, which protects against challenge with the parental tumor (Ostrand-Rosenberg *et al*., 1990; Clements *et al*., 1992; Armstrong *et al*., 1997, 1998b,a; Qi *et al*., 2000). Supratransfecting these engineered MHC class II-positive tumor cells with a gene for the Ii protein abrogated the vaccine potential of the modified cells (Clements *et al*., 1992; Armstrong *et al*., 1997).

We have shown that suppression of Ii protein synthesis by antisense methods enables MHC class II molecules to present TAA epitopes to helper T cells (Hillman *et al*., 2003a,b; Lu *et al*., 2003). Expressible Ii antisense reverse gene constructs (Ii-RGC) were engineered for inclusion into DNA vaccine vectors. These constructs were cloned into plasmids and/or engineered into adenoviruses to evaluate different methods of tumor gene transfection (Hillman *et al*., 2003a,b; Lu *et al*., 2003). The transfection of MHC class I and class II negative RM-9 cells, *in vitro*, using DNA plasmids encoding the genes for interferon-γ (pIFN-γ) and the MHC class II transactivator (pCIITA) caused upregulation of MHC class I molecules and MHC class II molecules, respectively (Hillman *et al*., 2003b). The Ii protein, coinduced by pCIITA transfection, was suppressed by an adenovirus encoding for an antisense reverse gene construct (Ii-RGC) (Hillman *et al*., 2003b). *In vivo*, the genes were delivered intratumorally in established RM-9 tumors using the plasmids pIFN-γ, pCIITA, pIi-RGC, and a subtherapeutic dose of a DNA plasmid encoding the interleukin-2 gene (pIL-2) used as an adjuvant cytokine. This treatment led to significant tumor growth inhibition but not to complete tumor regression (Hillman *et al*., 2003b). We showed that radiation of established tumors followed, a day later, by intratumoral injection of pIFN-γ, pCIITA, pIi-RGC, and pIL-2, resulted in complete tumor regression in more than 50% of the mice (Hillman *et al*., 2003b). Complete responders are defined by tumor regression and disappearance, and remaining tumor-free for more than 60–90 days of follow-up. Moreover, these complete responders were immune to rechallenge with parental tumor cells and demonstrated tumor-specific cytotoxic T cell activity (Hillman *et al*., 2003b). These data demonstrated that radiation enhanced the therapeutic effect of intratumoral gene therapy for *in situ* induction of a long-lasting tumor-specific immune response.

We have now investigated the requirement for each one of the four gene vectors, IFN-γ, CIITA, Ii-RGC, and IL-2, for the induction of the cancer vaccine when combined with prior tumor irradiation. We found that radiation and gene therapy using only the adjuvant plasmids IL-2, Ii-RGC, or both together did not cause complete tumor regression. Upregulation of MHC class I molecules with pIFN-γ, or class II molecules with pCIITA, respectively, was not sufficient to lead to a complete response even if supplemented with pIL-2 or pIi-RGC. An optimal and specific antitumor response was achieved in more than 50% of mice when, after tumor irradiation, tumor cells are converted *in situ* to the MHC class I+/class II+/Ii− phenotype by gene therapy with IFN-γ, CIITA, Ii-RGC and supplemented with adjuvant cytokine plasmid IL-2. Selective *in vivo* depletion of $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells abrogated the response to radiation and gene therapy confirming that these two T cell subsets play an essential role in the induction of complete antitumor immune response. Radiation caused significant debulking of the tumors *in situ* as demonstrated by significant colony formation inhibition of cells isolated from tumors at early time points between days 1–13 after radiation treatment. Apoptosis was documented histologically in these tumors as early as 1 day after radiation, at the time gene therapy was initiated. Complete tumor destruction by combined gene therapy was deter-

mined by lack of colony formation of cells isolated from these tumors and by histologic observation.

MATERIALS AND METHODS

Tumor model

The RM-9 murine prostate cancer cell line, provided by Dr. Timothy Tompson (Baylor College of Medicine, Houston, TX), was derived from independent primary prostate tumors induced in the Zipras/myc-9–infected mouse prostate reconstitution (MPR) model system using C57BL/6 mice as previously described (Thompson *et al*., 1989). Cells were maintained *in vivo* by serial subcutaneous passages and were also cultured *in vitro* in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillin G, 100 μg/ml streptomycin, and 10 m*M* HEPES buffer (Gibco BRL, Life Technologies, Grand Island, NY) (Hall *et al*., 1997; Nasu *et al*., 1999). Cells were passaged, *in vitro*, by trypsinization using 0.25% trypsin. For *in vivo* implantation, RM-9 cells were washed in Hanks' balanced salt solution (HBSS) and injected subcutaneously at 2×10^5 cells in 0.1 ml HBSS, in 4–6 week old C57BL/6 mice (Harlan Sprague Dawley Inc, Indianapolis, IN). For proper alignment in the radiation apparatus, cells were injected in the middle of the back, 1.5 cm from the tail (Hillman *et al*., 2003b). Mice were shaved prior to injection for accurate location of the injection site and for monitoring tumor growth. Mice were housed and handled in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The animal protocol was approved by the Wayne State University Animal Investigation Committee.

Gene expression vectors:

The plasmids pEF/Bsd/CIITA (pCIITA) and pcDNA (3)/IFN-γ (pIFN-γ) were constructed with cytomegalovirus (CMV) promoters based on constructs from Invitrogen (Carlsbad, CA) by standard molecular biology techniques. The plasmid Ii-RGC (pIi-RGC) was constructed by cloning an Ii gene fragment of base pairs from -92 to 97 (where A in the AUG start codon is position 1) into the RSV.5 vector in a reverse orientation, being driven by a RSV promoter to avoid promoter competition when large amounts of Ii-RGC were used (Hillman *et al*., 2003b). This construct was selected for our studies because it was more effective than the same construct driven by a CMV promoter (data not shown). The IL-2–containing plasmid (pIL-2), pNGVL-hIL-2 plasmid (CMV promoter/enhancer/intron A), was obtained from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, MI).

Radiation

An apparatus developed for radiotherapy of mouse prostate tumors (Hillman *et al*., 2001) was adapted for the radiation of subcutaneous tumors located in the middle of the back, 1.5 cm from the tail. Acrylic jigs were designed to place anesthetized mice in the supine position with their fore and hind limbs restrained by posts for reproducible and accurate positioning of the subcutaneous tumor on the back as described previously (Hillman *et al*., 2003b). Three jigs were positioned on an aluminum frame mounted on the x ray machine to irradiate three mice at a time. Lead shields of 6.4-mm thickness were designed with three cutouts for the three mice to expose the area of the tumor to photon irradiation while shielding the rest of the mouse body (Hillman *et al*., 2003b). The radiation dose to the tumor and the scattered dose to areas of the mouse outside of the radiation field were carefully monitored. Photon irradiation was performed with a Siemens Stabilipan X ray set (Siemens Medical Systems, Inc., Malvern, PA) operated at 250 kV, 15 mA with 1-mm copper filtration at a distance of 47.5 cm from the target.

Combination of radiation and intratumoral gene therapy with DNA plasmid vectors

Mice were injected subcutaneously with RM-9 cells at 2×10^5 cells in 0.1 ml HBSS. Mice with established tumors were treated on day 6 with selective tumor irradiation administered at a single dose of 8 Gy photons. One day later, on day 7, intratumoral injections of DNA plasmid vectors were initiated and continued on days 8, 9, and 10 as previously described (Hillman *et al*., 2003b). CIITA, IFN-γ, and IL-2 DNA plasmids were injected at a dose of 3 μg per injection per day while Ii-RGC DNA plasmid was injected at 31 μg per injection per day. We used approximately 10 times more Ii-RGC than CIITA in order to ensure that each cell transfected with a CIITA gene was also transfected with Ii-RGC, and to ensure that there would be sufficient suppression of the Ii protein in light of Ii induction caused by CIITA. A total of 40 μg of plasmid were injected per mouse, and the total amount of plasmid DNA was adjusted when needed using empty plasmid DNA to result in the same total DNA for all groups. Plasmids vectors were mixed with a liposome formulation of cationic lipid DMRIE [1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide/cholesterol] (DMRIE-C, Gibco, Life Technologies) 2–4 min prior to injection at a ratio of 1:5 w/w, DMRIE/DNA. Experimental groups were treated either with intratumoral PBS or tumor irradiation and intratumoral PBS, or tumor irradiation and various combinations of plasmids. Mice were monitored for tumor growth and survival. Tumors were measured in three dimensions, three times per week, with a caliper. Tumor volume was calculated using the equation: $0.5236 \times \text{length} \times \text{width} \times \text{height}$. In all experiments, when tumors reached 1.5 cm in greatest diameter or 1 cm with ulceration, mice were sacrificed in accordance with animal facilities regulations. Mice with no evidence of tumor by day 64–70 underwent rechallenge with 1×10^5 parental RM-9 tumor cells injected subcutaneously in the opposite flank; as a control, three naïve mice also underwent challenge in this manner.

In vivo *depletion of $CD4^+$ or $CD8^+$ T cell subsets*

Mice were injected subcutaneously with RM-9 cells at 2×10^5 cells in 0.1 ml HBSS. On days 1, 4, 6, and 12, mice were injected with either anti-CD4 monoclonal antibody (mAb) or anti-CD8 mAb. To deplete $CD4^+$ T cells, 0.1 ml ascites fluid of GK 1.5 mAb was injected intraperitoneally. To deplete $CD8^+$ T cells, mice were injected intraperitoneally with 0.5 ml hy-

bridoma culture supernatant of Ly-2 mAb that was purified using the Montage Antibody Purification Kit with PROSEP-A (Millipore, Billerica, MA). On day 6, tumor irradiation was administered at 8 Gy photons followed on days 7–10 by daily intratumoral injections of pCIITA + pIFN-γ + pIi-RGC + pIL-2 plasmid combinations as described above. Depletion of T cells was monitored on days 7, 13, and 27 post-cell injection, by immunofluorescent staining of mouse splenocytes with specific antibodies as previously described (Younes *et al*., 1995). Splenocytes (10^6) were washed in phosphate-buffered saline (PBS) containing 0.1% sodium azide and 0.1% fetal calf serum (FCS) and then labeled with antibodies for 30 min at 4°C. The mAbs anti-L3T4 conjugated to phycoerythrin (PE) and anti-Lyt-2 conjugated to fluorescein isothiocyanate (FITC) were used for $CD4^+$ and $CD8^+$ T cells respectively (Caltag Laboratories, Burlingame, CA). Gates were set for nonspecific binding using cells labeled with the isotypes rat IgG_{2b}-FITC and rat IgG_{2b}-PE (Caltag Laboratories). Cells were analyzed on a FACScan flow cytometer.

Tumor processing for cell viability and colony formation assay

Tumors were resected at different time points, weighed, and processed into a single cell suspension. Tumors were minced into small pieces and dissociated by enzymatic digestion with 0.4 mg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO) in RPMI-1640 medium supplemented with 2 m*M* glutamine and 100 U/ml penicillin/streptomycin. Tumor digestion was done at 37°C for 2 hr with stirring, and then cells were filtered through a wire mesh. The cell suspension was washed twice in medium. The number of viable cells was determined by trypan blue exclusion. Cells were plated for colony assay in triplicates in 6-well plates at a concentration of 3000 cells per well for cells from control tumors, radiation-, or plasmid-treated tumors, and 1000 cells per well for radiation- plus plasmid-treated tumors in 2 ml CM. After 8 days incubation at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator, colonies were fixed and stained in 2% crystal violet in absolute ethanol, then counted. The plating efficiency was calculated for each well by dividing the number of colonies by the original number of cells plated. The surviving fraction was normalized to the cell plating efficiency of control cells by dividing the plating efficiency of treated cells by that of control cells.

Histology

Tumors were resected at different time points and processed for histology studies. Tumors were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E). To detect apoptotic cells, paraffin-embedded sections were pretreated with proteinase K (20 μg/ml) for 15 min and stained using an In Situ Cell Death Detection Kit peroxidase POD (TUNEL) according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Slides were counterstained with Mayer's hematoxylin.

Statistical analysis

To compare the proportion of mice with complete tumor regression, the χ^2 test was used at the statistical significance level of 0.05.

RESULTS

Radiation and induction of the MHC class I+/class II+/Ii- phenotype for optimal antitumor response in RM-9 tumors

We previously demonstrated that an optimal antitumor response induced by intratumoral gene therapy was obtained only when radiation was given to the tumor selectively 1 day prior to gene therapy (Hillman *et al*., 2003b). The gene therapy, which was used to convert the tumor cells *in situ* into a potent cancer vaccine, consisted of a mixture of the four DNA plasmid vectors pCIITA, pIFN-γ, pIi-RGC, and pIL-2. In order to dissect the relative roles of each plasmid in inducing the cancer vaccine response, we have now treated established RM-9 tumors of 0.3–0.4 cm with 8 Gy radiation followed a day later by intratumoral injection of various combinations of plasmids given once per day for 4 consecutive days. In repeated experiments, treatment of tumors with PBS, or with radiation and PBS, did not lead to complete tumor regression (Table 1), as shown previously (Hillman *et al*., 2003b). Treating tumors with radiation followed by empty plasmid injections also did not cause complete tumor regression (Table 1, *I*). Single-plasmid gene therapy using pIL-2 or pIi-RGC combined with tumor irradiation also did not result in a complete antitumor immune response (Table 1, *I*). These data confirm that pIL-2, *per se*, is not therapeutic at the low dose of 3 μg used in these studies. As expected, pIi-RGC by itself is not sufficient to cause an effect on tumor growth in RM-9 cells negative for Ii and MHC class II molecules. Combining radiation with pIi-RGC and pIL-2 led to one of six mice having complete tumor regression; however, this mouse was not immune to RM-9 rechallenge, ruling out induction of immune response with specific tumor immunity by this treatment (Table 1, *I*).

We have shown that pIFN-γ transfection of RM-9 cells induces cell surface expression of MHC class I molecules (Hillman *et al*., 2003b). In order to address whether induction of MHC class I molecules is sufficient to get a therapeutic effect, tumors were treated with radiation followed by a mixture of pIFN-γ and pIi-RGC. No complete responders were observed in eight treated mice showing that upregulation of MHC class I molecules by pIFN-γ was not sufficient to induce a complete tumor response and that pIi-RGC also did not affect this response as could be expected (Table 1, *II*). The addition of pIL-2 led to one responder out of eight, this finding might be incidental as found with radiation plus pIi-RGC plus pIL-2 (Table 1, *II*).

We have shown that pCIITA transfection of RM-9 cells causes upregulation of MHC class II cell surface molecules and intracellular Ii protein (Hillman *et al*., 2003b). To test whether induction of MHC class II molecules and suppression of Ii are sufficient to get a therapeutic effect, tumors were treated with radiation followed by a mixture of pCIITA and pIi-RGC. Upregulating MHC class II molecules by pCIITA and decreasing Ii protein by pIi-RGC were not sufficient to induce a complete tumor response (Table 1, *III*). However, addition of an adjuvant dose of pIL-2 cytokine induced a complete and significant antitumor response in 30% of the mice compared to the same treatment with pIL-2 alone ($p < 0.001$). This antitumor response was the result of a specific immune response as con-

Table 1. Radiation and Induction of the MHC Class I+/MHC Class II+/Ii− Phenotype Provide Optimal Antitumor Response to RM-9 Tumors

	Tumor-free mice	
Treatment	*Post-treatment*	*Post-RM-9 challenge*
PBS control	0/20[a]	NA
Radiation	0/20[a]	NA
I. Adjuvant plasmids		
Radiation + empty plasmid	0/5	NA
Radiation + pIL-2	0/5	NA
Radiation + pIi-RGC	0/7	NA
Radiation + pIi-RGC + pIL-2	1/6	0/1
II. MHC Class I+		
Radiation + pIFN-γ + pIi-RGC	0/8	NA
Radiation + pIFN-γ + pIi-RGC + pIL-2	1/8	NT
III. MHC Class II+		
Radiation + pCIITA + pIi-RGC	0/8	NA
Radiation + pCIITA + pIi-RGC + pIL-2	4/13[b]	4/4
IV. MHC Class I+/Class II+		
Radiation + pCIITA + pIFN-γ	0/5	NA
Radiation + pCIITA + pIFN-γ + pIi-RGC	1/7	1/1
Radiation + pCIITA + pIFN-γ + pIL-2	3/11[b]	3/3
Radiation + pCIITA + pIFN-γ + pIi-RGC + pIL-2	11/21[b]	11/11

[a]In control PBS and radiation groups, 5 mice per group were used in each of the 4 experiments resulting in no antitumor response in a total of 20 mice.

[b]In these radiation + plasmids group, data from 2–3 repeated experiments were compiled.

Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 6. On day 7, intratumoral plasmid therapy with various plasmid combinations was initiated for 4 consecutive days as detailed in Materials and Methods. The proportion of tumor-free mice at the end of the observation period, by day 64–70 after radiation and plasmid therapy is presented. Tumor-free mice and naïve mice were challenged with RM-9 cells at that time. The proportion of challenge-tumor free mice after 3–4 weeks post-tumor challenge is reported. These data are compiled from four separate experiments.

MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

firmed by rejection of RM-9 challenge in the four complete responders of 13 treated mice (Table 1, *III*).

Treatment of mice with tumor irradiation followed by pIFN-γ and pCIITA to upregulate MHC class I molecules and class II molecules associated with Ii synthesis was not sufficient to cause a complete response (Table 1, *IV)*. Addition of pIi-RGC to decrease Ii synthesis induced a complete specific antitumor response in one of seven mice (14% response). Addition of pIL-2 to pCIITA and pIFN-γ caused a complete antitumor response in 27% of the mice confirming a role for IL-2 to act as an adjuvant to enhance an immune response triggered by tumor cells expressing MHC class I and class II molecules (Table 1, *IV)*. However, when pIi-RGC was added to the mixture of pCIITA plus pIFN-γ plus pIL-2, to decrease Ii synthesis, the number of mice responding with complete tumor regression was consistently increased resulting in a complete and lasting response over 60 days in more than 50% of the mice (Table 1, *IV)*. Comparisons between treatment groups showed that addition of pIi-RGC and pIL-2 to pCIITA and pIFN-γ was significant ($p < 0.005$) and addition of pCIITA to pIFN-γ plus pIi-RGC plus pIL-2 was significant ($p < 0.05$). The complete tumor responses observed in series *IV* of *in situ* induction of MHC class I+/class II+ combined with adjuvant plasmids were caused by a specific antitumor immune response because all responding mice rejected RM-9 tumor cell rechallenge administered on day 64 (Table 1, *IV)*. Mice rejecting challenge tumors were clear of tumors during a 3–4 week period. In contrast, all naïve mice developed RM-9 tumors by 7–10 days after challenge with RM-9 cells.

Effect of in vivo *depletion of CD4$^+$ or CD8$^+$ T cells on the antitumor response induced by radiation and gene therapy in RM-9 tumors*

To assess the role of CD4$^+$ helper T cells and CD8$^+$ cytotoxic T cells, mice were injected with mAb specific to these subpopulations before and after treatment with tumor irradiation and pCIITA plus pIFN-γ plus pIi-RGC plus pIL-2 intratumoral gene therapy (as detailed in Materials and Methods). Tumor growth was inhibited by radiation and gene therapy by more than approximately 20 days compared to control tumors (Fig. 1A and 1B), as previously described (Hillman *et al*., 2003b). Tumor progression was observed in 6 of 12 mice by day 30 while the remaining 6 of 12 mice showed tumor re-

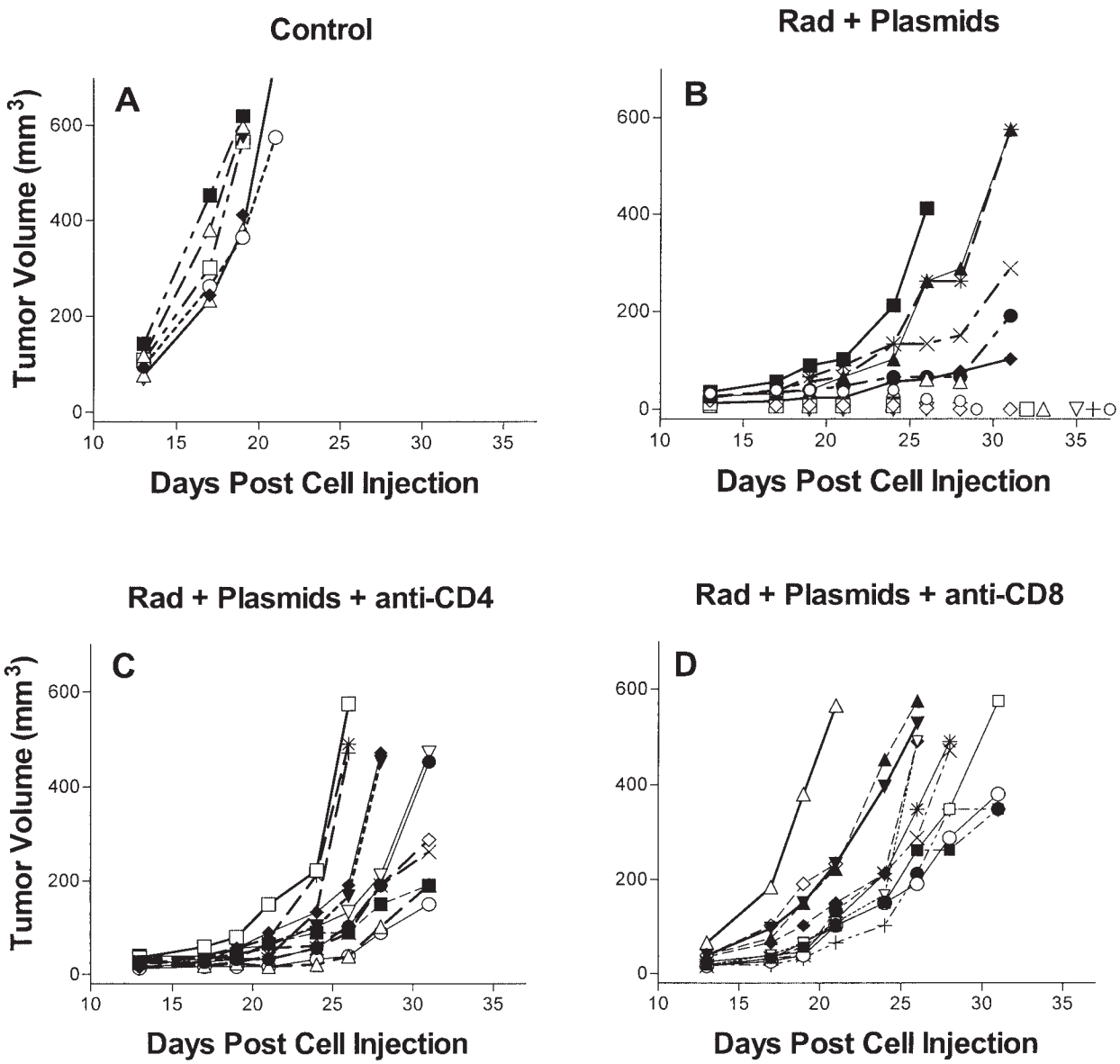


FIG. 1. Growth of RM-9 tumors in mice depleted of T cells and treated with irradiation and gene therapy. Mice were injected subcutaneously with RM-9 cells and treated with intraperitoneal injections of GK 1.5 anti-CD4 monoclonal antibody (mAb(or Ly-2 anti-CD8 mAb before and after gene therapy on day 1, 4, 6, and 12. On day 6, mice were treated with 8 Gy tumor irradiation followed on days 7–10 by daily intratumoral injections of pCIITA plus pIFN-γ plus pIi-RGC plus pIL-2 plasmids. **A**: Control mice treated with phosphate buffered saline (PBS). **B**: Mice treated with tumor irradiation plus gene therapy. **C**: Mice pretreated with anti-CD4 mAb then with radiation plus gene therapy. **D**: Mice pretreated with anti-CD8 mAb then with radiation plus gene therapy. In panels (**B**), (**C**), and (**D**), the tumor volume of 12 individual mice is represented each by a different symbol. Complete tumor regressions were observed in 6 of 12 mice treated with radiation and gene therapy (**B**) compared to 0 of 12 in mice depleted of either $CD4^+$ T cells (**C**) or $CD8^+$ T cells (**D**).

gression that was consistent with our previous findings of approximately 50% response (Fig. 1B, Table 1; Hillman *et al*., 2003b). In treatment groups receiving either anti-CD4 mAb or anti-CD8 mAb, tumor growth was inhibited initially probably due to the radiation effect, but after day 20, all tumors progressed rapidly to large sizes (Figure 1 C, D). Tumor regression was observed in 0 of 12 mice treated with anti-CD4 mAb and in 0 of 12 mice treated with anti-CD8 mAb compared to 6 of 12 mice treated with radiation plus gene therapy but not depleted of T cells. Therefore, the antitumor response mediated by tumor irradiation and gene therapy was abrogated by depletion of $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells prior and after therapy. A second identical experiment showed reproducibility of our findings in which 4 of 10 mice had complete tumor regression after tumor irradiation and gene therapy while 0 of 10 and 1 of 8 had tumor regression in groups treated

with anti-CD4 and anti-CD8 mAbs, respectively. In both experiments, immune monitoring of $CD4^+$ T cell subsets or $CD8^+$ T cell subsets on days 7, 13, and 27 by immunofluorescent staining of mouse splenocytes, confirmed the depletion of these populations. $CD4^+$ T cells were completely depleted *in vivo* during and after treatment with plasmids for at least 4 weeks (data not shown). Similarly, depletion of $CD8^+$ T cells was also complete for several days during and after treatment with plasmids and lasted for 4 weeks (data not shown). We found that by day 42, $CD4^+$ T cells and $CD8^+$ T cells started to regenerate. The percent of $CD4^+$ T cells and $CD8^+$ T cells was comparable in naïve mice, RM-9–bearing mice and mice treated with tumor irradiation and gene therapy without T cell depletion and was in the range of 16–20% for $CD4^+$ T cells and 9–12% for $CD8^+$ T cells.

Viability and division ability of cells isolated from RM-9 tumors treated with radiation and gene therapy

To investigate the contribution of radiation to the extent of cell killing prior to and after gene therapy, established tumors were treated with radiation and pCIITA plus pIFN-γ plus pIi-RGC plus pIL-2 intratumoral gene therapy or each therapy alone (as detailed in Materials and Methods). On days 1, 5, 8, and 13 postradiation (corresponding to days 0, 1, 4, and 9 after gene therapy), tumors were resected and weighed. One tumor from each group was fixed in formalin for histology studies described below and one tumor was dissociated into single-cell suspension for cell count and colony formation assay as detailed in Materials and Methods. These kinetic studies showed that tumors grew rapidly in control nonirradiated tumors while radiation inhibited the growth of the tumor up to 13 days after tumor irradiation (Fig. 2A). After plasmid therapy, growth of the tumors resumed 4 days after the end of gene therapy while radiation combined with plasmid therapy decreased the tumor burden by 4 days after gene therapy with minimal measurable nodules, a lasting effect seen by day 9 after gene therapy or day 13 after radiation in contrast to tumors treated with plasmids alone (Fig. 2A). The number and viability of the tumor cells isolated from these tumors followed the same pattern with rapid increase in the number of viable cells in control tumors and relatively lower number of cells in radiation-treated tumors for up to 13 days after radiation (Fig. 2B). Already by 1 day after radiation, the recovery of viable tumor cells was five times less than in control tumor. An increase in the number of viable cells was observed 4 days after plasmid therapy while the number of viable cells isolated from radiation plus plasmid-treated tumors remained low (< 0.4–0.6×10^5 per tumor) (Fig. 2B).

To determine the division ability of the cells isolated from treated tumors, cells were plated in an 8-day colony formation assay. The surviving fraction showed that cells isolated from radiation treated tumors, 1 day after radiation, had approximately 60% inhibition in their ability to form colonies relative to control tumors (Fig. 2C). This inhibition remained in the range of 40–50% over 13 days after radiation. These data corroborate the findings of the kinetics of tumor growth and viability of the cells over 13 days remaining at a low level after radiation. On days 4 and 9 after plasmid therapy an inhibition of 30–40% colony formation was observed relative to control (Fig. 2C). Treatment with radiation and plasmids almost completely abrogated the ability of tumor cells to divide, corroborating the low tumor weight and the low number of cells recovered from these tumors (Fig. 2C). These data were consistently reproduced in a second experiment.

Histologic evaluation of RM-9 tumors treated with radiation and gene therapy

To determine the *in situ* alterations induced by radiation and gene therapy and the extent of tumor destruction compared to each treatment alone, separate tumors resected from the experiment described above and depicted in Figure 2 were processed for histologic studies. Tumor sections were stained with H&E and others were stained using the TUNEL assay as detailed in Materials and Methods. Untreated RM-9 tumors presented as sheets of pleomorphic epithelial cells, with large nuclei and prominent nucleoli (Fig. 3A), and few apoptotic cells (Fig. 3B). Already 1 day after radiation, areas of focal necrosis and apoptotic cells were scattered in the tumor nodules as seen by H&E staining (Fig. 3C) and confirmed by TUNEL staining (Fig. 3D). An increase in fibrosis, inflammatory infiltrates, including polymorphonuclear cells (PMN) and lymphocytes, and focal hemorrhages were observed at 5–13 days postradiation, however, approximately 50–70% of the tumor cells looked viable. A larger number of giant cells tumors were seen that are characteristic of radiation induced cell alterations. After plasmid therapy, areas of tumor destruction at the periphery of the tumor nodules were observed with apoptotic cells, infiltration of inflammatory cells and vascular damage whereas 60–70% of viable tumor was seen in the center of the tumor (Fig. 3E). By day 9 after the end of gene therapy, most of the tumor showed little apoptosis (Fig. 3F). In contrast, treatment with radiation and plasmid therapy resulted in small tumor nodules, showing significant changes already at 1 day after the end of gene therapy that became prominent at 4 and 9 days after therapy. Tumor presented with large areas of necrosis associated with cell debris, apoptotic bodies, fibrosis, and focal hemorrhages (Fig. 3H). Few or no viable tumor cells were observed as confirmed by the large number of stained apoptotic cells in TUNEL (Fig. 3G). A heavy infiltration of inflammatory cells in the periphery and inside the tumor nodule consisted of lymphocytes, histiocytes and neutrophils. These data were consistently reproduced in a second experiment.

DISCUSSION

We have developed a novel approach combining selective tumor irradiation with gene-mediated immunotherapy that converts tumor cells, *in situ*, into a curative cancer vaccine in the murine RM-9 prostate tumor model. We showed that intratumoral gene therapy of established RM-9 subcutaneous tumor nodules with plasmid cDNAs coding for the MHC class I inducer IFN-γ, the MHC class II inducer CIITA and an Ii suppressor gene, to upregulate MHC class I and class II molecules and suppress the Ii invariant chain, transiently inhibited tumor growth (Hillman *et al*., 2003b). This effect suggested that this

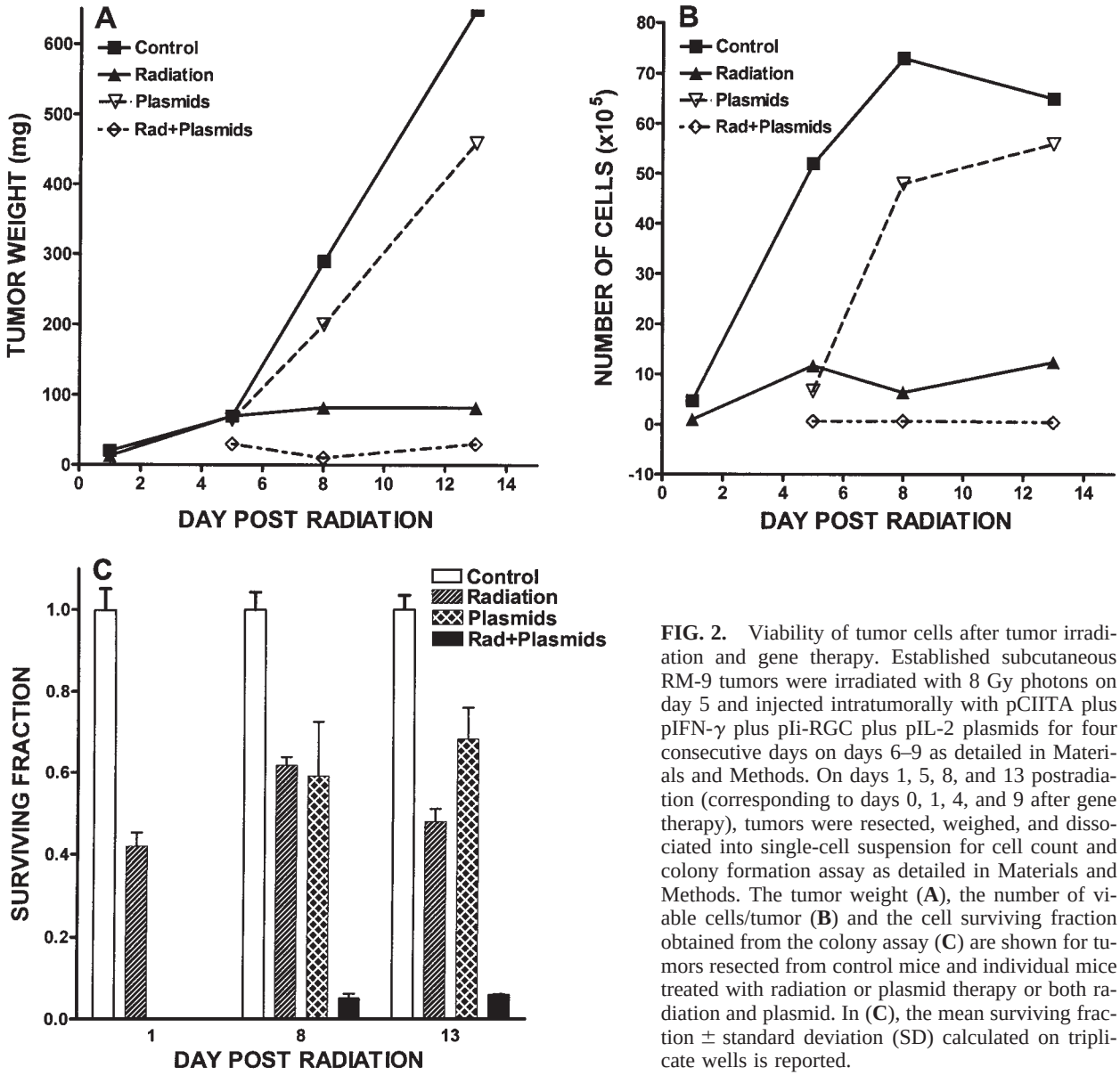
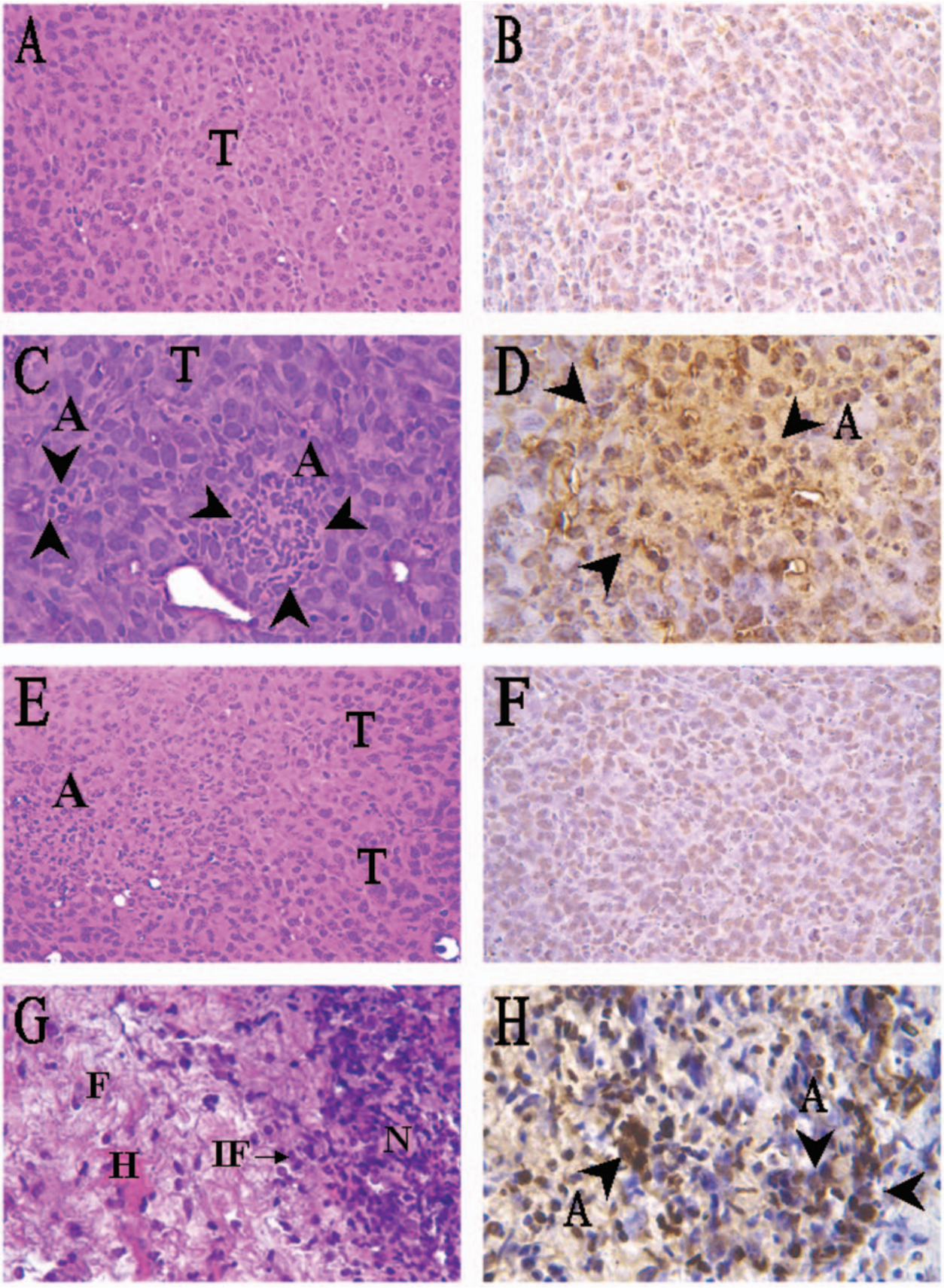


FIG. 2. Viability of tumor cells after tumor irradiation and gene therapy. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 5 and injected intratumorally with pCIITA plus pIFN-γ plus pIi-RGC plus pIL-2 plasmids for four consecutive days on days 6–9 as detailed in Materials and Methods. On days 1, 5, 8, and 13 postradiation (corresponding to days 0, 1, 4, and 9 after gene therapy), tumors were resected, weighed, and dissociated into single-cell suspension for cell count and colony formation assay as detailed in Materials and Methods. The tumor weight (**A**), the number of viable cells/tumor (**B**) and the cell surviving fraction obtained from the colony assay (**C**) are shown for tumors resected from control mice and individual mice treated with radiation or plasmid therapy or both radiation and plasmid. In (**C**), the mean surviving fraction ± standard deviation (SD) calculated on triplicate wells is reported.

FIG. 3. Histology of RM-9 tumors treated with radiation and plasmid gene therapy. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 5 and injected intratumorally with pCIITA plus pIFN-γ plus pIi-RGC plus pIL-2 plasmids for 4 consecutive days on days 6–9 as detailed in Materials and Methods. Tumors were resected at different time points and tumor sections were stained with hematoxylin and eosin (H&E; **A**, **C**, **E**, **H**) or for apoptosis with TUNEL In Situ Cell Death Detection Kit peroxidase POD (**B**, **D**, **F**, **G**) as described in Materials and Methods. The main findings were labeled on the prints with T for tumor, A for apoptosis, H for hemorrhages, N for necrosis, F for fibrosis, and IF for inflammatory cells. **A**: Untreated tumor, sheets of pleomorphic epithelial cells with frequent mitosis. **B**: Untreated tumor stained with TUNEL showing few stained cells. **C**: Radiation treated tumor on day 1 postradiation, note focal areas of apoptotic cells as confirmed by TUNEL staining in (**D**). **E**: Tumor treated with plasmids at 4 days after the end of gene therapy showing areas of tumor destruction and areas of viable tumor. **F**: Tumor treated with plasmids at 9 days after the end of gene therapy stained with TUNEL confirming viable tumor and minimal apoptosis. **G**: Radiation- plus plasmid-treated tumor at 4 days after the end of gene therapy showing large areas of necrosis with extensive apoptosis, focal hemorrhages, fibrosis, and inflammatory cells. **H**: Radiation- plus plasmid-treated tumor at 9 days after the end of gene therapy stained with TUNEL exhibiting large numbers of apoptotic cells. All magnifications, ×50.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gene therapy approach induced an immune response, but that this response was not sufficient to eradicate the poorly immunogenic and rapidly growing tumors in the RM-9 model. However, when radiation was applied to RM-9 tumors 1 day prior to intratumoral gene therapy, complete tumor regressions occurred in approximately 50% of the mice (Hillman *et al*., 2003b). These complete responders, rendered tumor-free by the combined therapy, were immune to rechallenge with parental tumor and demonstrated specific cytotoxic T cell activity (Hillman *et al*., 2003b). These data confirm that tumor irradiation in conjunction with gene-mediated immunotherapy induced a significantly stronger antitumor immune response resulting in eradication of the tumor nodule and long-lasting tumor immunity. This effect was obtained when gene therapy was administered by injections of a mixture of the four individual plasmid vectors, CIITA, IFNγ, Ii-RGC, and IL-2 in liposome formulation.

To determine the role of each plasmid in induction of the antitumor immune response, we have treated established RM-9 subcutaneous tumors with radiation followed a day later by intratumoral plasmid injections using various combinations of the four plasmids. We found that radiation and gene therapy using adjuvant plasmids IL-2 or Ii-RGC or both were ineffective at causing complete tumor regression. These data confirm that pIL-2 is not therapeutic at the low dose of 3 μg used in these studies in contrast to the 50-μg tumoricidal dose used in other studies (Saffran *et al*., 1998). As expected, pIi-RGC by itself is not sufficient to cause an effect on tumor growth in RM-9 cells negative for Ii and MHC class II molecules. Similarly, upregulation of MHC class I molecules by IFN-γ plasmid was not sufficient to lead to a complete response even when IL-2 plasmid was added. These data indicate that tumor cells expressing only MHC class I molecules presenting TAA and not class II molecules cannot act as APCs to mediate a strong antitumor immune response via stimulation of $CD8^+$ cytotoxic T cells. However, upregulation of MHC class II molecules by the CIITA plasmid and inhibition of Ii synthesis by Ii-RGC caused complete tumor regression associated with specific immunity in 30% of the mice but only when supplemented with low doses of IL-2 plasmid. These data suggest the importance of stimulation of $CD4^+$ T cells by novel endogenous TAA presented by MHC molecules (Hillman *et al*., 2004a; Xu *et al*., 2004). IL-2 may play a role in regulating the T cell activation. Induction of MHC class I+/class II+ by mixed CIITA and IFN-γ plasmids was not effective but addition of Ii-RGC or IL-2 plasmids led to 14–27% complete responders.

The combination of the four IFN-γ, CIITA, Ii-RGC, and IL-2 plasmids with tumor irradiation consistently led to a specific antitumor immune response associated with long-lasting complete tumor regression and immunity to tumor rechallenge in more than 50% of the mice. These data demonstrate that an optimal and specific antitumor immune response is achieved in mice treated with tumor irradiation followed by gene therapy, with a combination of the four plasmids pCIITA, pIFN-γ, pIi-RGC, and pIL-2, converting the tumor cells *in situ* to the MHC class I+/class II+/Ii− phenotype. Such a phenotype helped by the adjuvant cytokine IL-2, probably acting as the second signal for T cell stimulation in addition to MHC presenting tumor peptides to the T cell receptor, converts the cells into a cancer vaccine. IL-2 may also act to sustain and enhance the T cell activation triggered by modified tumor cells as previously shown in other studies (Kim *et al*., 2001).

Modified MHC class I+/class II+/Ii− cells allow for presentation of endogenous tumor antigens by MHC class II molecules to $CD4^+$ T helper cells. We have now demonstrated that these helper T cells play an essential role in the induction of a complete antitumor immune response triggered by our combined radiation and gene therapy approach. Depletion of $CD4^+$ T helper cells *in vivo* prior to and during radiation/gene therapy treatment abrogated the complete antitumor response induced by radiation and plasmid therapy. Depletion of $CD8^+$ cytotoxic T cells also resulted in the elimination of complete responders. Immune monitoring of $CD4^+$ T cells and $CD8^+$ T cells confirmed that these cells were depleted before therapy and for at least 4 weeks after therapy, a crucial time for the antitumor immune response to develop. These data demonstrate that the antitumor effect observed after tumor irradiation and genetic modification of tumor cells to the MHC class I+/class II+/Ii− phenotype is mediated by induction of a robust antitumor immune response dependent on both $CD4^+$ helper and $CD8^+$ cytotoxic T cell subsets.

These studies provide a direct confirmation that creation of the MHC class I+/II+/Ii− phenotype to allow tumor cells simultaneously present both MHC class I- and class II-restricted TAA epitopes has the potential to trigger a robust and specific antitumor immune response able to eradicate the tumor. Induction of MHC class II molecules and Ii by CIITA together with suppression of Ii by Ii-RGC, is a clinically practical method because both CIITA and Ii genes are monoallelic (Hillman *et al*., 2004a; Xu *et al*., 2004.). Transfecting the tumors of each patient with genes for his or her own MHC class II alleles is not clinically practical in large numbers of patients.

The mechanisms by which tumor irradiation enhances the therapeutic efficacy of intratumoral gene therapy, for *in situ* conversion of tumor cells into a cancer vaccine, is a major focus of our work. Two possible mechanisms for radiation enhancement of gene therapy are the DNA-damaging and tissue-debulking effects that slow tumor growth and give time for the immune response to become effective (Dezso *et al*., 1996; Hillman *et al*., 2003b). We have now shown that as early as 1 day after tumor irradiation, at the time of initiation of plasmid injections, there are already five times fewer viable cells isolated from irradiated tumors compared to control tumors. A 60% inhibition in the division ability of these *in situ* irradiated tumor cells, relative to cells from control tumors, was measured in a colony formation assay. These data confirm that at the time gene therapy is initiated in the irradiated tumor nodules, there is a significantly lower number of functional cells, increasing the probability of tumor cell transfection and consistent with the debulking effect of radiation. Moreover, this effect persists for almost 2 weeks after radiation as seen in inhibition of tumor growth, lower number of viable cells, and decrease in division ability. These findings were confirmed by the histologic observation of irradiated tumors presenting with focal areas of apoptotic cells as soon as 1 day postradiation. By 2 weeks after radiation, remaining viable tumor was observed, consistent with subsequent tumor regrowth. As shown in our previous studies, inhibition of growth of irradiated tumors was transient and growth resumed after 2 weeks after radiation corroborating the present findings (Hillman *et al*., 2003b). Monitoring of

cells isolated from plasmid treated tumors also showed inhibition of 30–40% of the ability to form colonies, consistent with the transient inhibition observed in tumor growth (Hillman *et al*., 2003b). In contrast, the effect of gene therapy combined with prior tumor irradiation was more drastic and observed already just at one day after the end of gene therapy with a decrease in tumor size, recovery of few viable cells with limited or no ability to divide in the colony assay. This dramatic inhibition of tumor growth persisted and was confirmed by the histologic observation of complete destruction of tumor cells. Tumor nodules showed extensive necrosis, apoptosis, and fibrosis.

Another possibility for mechanism of interaction between the two modalities is that radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity that can be readily activated by *in situ* gene therapy (Dezso *et al*., 1996). In this study, we showed that radiation caused vascular damage and infiltration of PMN and lymphocytes in RM-9 tumors confirming mobilization of inflammatory cells. A large influx of inflammatory cells consisting of lymphocytes, neutrophils, and histiocytes was observed in tumors treated with radiation and plasmid therapy localized both at the periphery and inside the nodules in areas of fibrosis and necrosis. This is consistent with our findings of induction of antitumor immune response associated with T cell activity as shown in the T cell depletion experiments (Fig. 1) and cytotoxic T cell activity previously demonstrated (Hillman *et al*., 2003b). Interestingly, an influx of inflammatory cells associated with tumor destruction was also seen in nonirradiated plasmid-treated tumors, but it was localized only at the periphery of the tumor while tumor in the center of the nodule looked viable and resulted in tumor regrowth. Radiation might enhance the permeability of the tumor allowing a greater influx of activated immune cells inside the nodules.

Radiation could increase gene transduction efficiency and duration of expression of surviving tumor cells, thus improving efficiency of *in situ* genetic modification leading to an immune response that eradicated remaining tumor cells. Radiation improved the transfection efficiency of plasmid DNA in normal and malignant cells, *in vitro*, resulting from radiation-induced DNA breaks and DNA repair mechanisms (Zeng *et al*., 1997). These studies showed that radiation followed by plasmid or adenoviral transfection enhanced integration of the transgene (Stevens *et al*., 1996; Zeng *et al*., 1997). Other recent studies also showed that ionizing radiation increased adenoviral vector uptake and improved transgene expression in tumor xenografts (Zhang *et al*., 2003). We found that tumor irradiation also enhanced the anti-tumor response mediated by intratumoral injections of the IL-2 adenovector (Ad-IL-2) in the Renca murine renal adenocarcinoma (Hillman *et al*., 2004b). Our preliminary studies in the RM-9 and Renca models, using intratumoral injections of Ad-IL-2, show that radiation potentiates the genetic modification of the tumor cells by increasing both the level and duration of expression of transfected genes (unpublished observations). Our studies and others indicate that radiation improves gene transfection efficiency. Radiation might also limit suppressive immunoregulatory T cells; previous studies in the RM model have shown evidence that RM tumors are immunosuppressive and induce tumor-specific CD4+ regulatory T cells (Griffith *et al*., 2001).

We are pursuing additional studies to clarify further the mechanisms by which radiation improves the efficacy of gene therapy, to optimize the conditions of radiation/plasmid combination to increase therapeutic efficacy, and to test this novel approach in orthotopic transplants for both local tumor eradication and control of spontaneous metastases. In addition, we are addressing the question as to why 100% of the mice are not cured. Possibly, we are already at nearly optimal conditions for our therapy and failure to cure lies in issues of T cell immunoregulatory function, tumor cell sequestration, and protective fibrosis. One might be able to anticipate in which mice cures will not occur by polymerase chain reaction (PCR) analysis of cytokine transcripts of defined subsets of tumor-infiltrating lymphocytes.

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Address reprint requests to:
Gilda G. Hillman, Ph.D.
Department of Radiation Oncology
Wayne State University School of Medicine
Karmanos Cancer Institute
Hudson-Webber Building, Room 515, 4100 John R
Detroit, MI 48201

E-mail: hillmang@karmanos.org.

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Suppression of major histocompatibility complex class II-associated invariant chain enhances the potency of an HIV gp120 DNA vaccine

Xueqing Lu, Shuzhen Wu, Catherine E. Blackwell, Robert E. Humphreys, Eric von Hofe and Minzhen Xu

Antigen Express, Inc., Worcester, MA, USA

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Correspondence: Minzhen Xu, Antigen Express, Inc., 100 Barber Avenue, Worcester, MA 01606–2758, USA.
Email: mxu@antigenexpress.com

Summary

One function of the major histocompatibility complex (MHC) class II-associated invariant chain (Ii) is to prevent MHC class II molecules from binding endogenously generated antigenic epitopes. Ii inhibition leads to MHC class II presentation of endogenous antigens by APC without interrupting MHC class I presentation. We present data that *in vivo* immunization of BALB/c mice with HIV gp120 cDNA plus an Ii suppressive construct significantly enhances the activation of both gp120-specific T helper (Th) cells and cytotoxic T lymphocytes (CTL). Our results support the concept that MHC class II-positive/Ii-negative (class II^+/Ii^-) antigen-presenting cells (APC) present endogenously synthesized vaccine antigens simultaneously by MHC class II and class I molecules, activating both $CD4^+$ and $CD8^+$ T cells. Activated $CD4^+$ T cells locally strengthen the response of $CD8^+$ CTL, thus enhancing the potency of a DNA vaccine.

Keywords: DNA vaccine; HIV gp120; Ii suppression; MHC class II

Introduction

While DNA vaccines induce cytotoxic T lymphocyte (CTL) activity successfully and are considered to be generally safe and economic, they often stimulate a relatively poor immune response. Investigators have sought to enhance the immunogenicity of DNA vaccines using a variety of methods. These techniques include: inoculation with genes encoding costimulatory molecules,[1] enhancing the *in vivo* transfection efficiency by mixing DNA with cationic lipids,[2] coating DNA onto microparticles such as poly(lactide-coglycolide)[3] and *in vivo* electroporation.[4] Other methods that are being explored to enhance vaccine gene expression include using a gene gun to deliver DNA vaccines directly into cells,[5] the addition of CpG motifs to plamsids to generate innate immune stimuli[6,7] and the development of different prime/boost regimes using DNA/virus, DNA/protein and DNA/peptides.[8–14] In addition, cytokine genes, such as granulocyte–macrophage colony-stimulating factor (GM-CSF), have been used in DNA vaccine regimes to augment DNA vaccine efficiency.[6,15–17] Our studies add to the work of the above investigators, with a novel and potentially clinically useful method to enhance the potency of DNA vaccines.

$CD4^+$ T cell activation plays an important role in the enhancement of DNA vaccine efficacy.[18] We have developed a vaccine strategy, based on suppression of the expression of major histocompatibility complex (MHC) class II associated invariant chain protein (Ii), that augments $CD4^+$ T cell activation by endogenously synthesized antigens. The Ii protein normally binds to MHC class II molecules in the endoplasmic reticulum (ER), blocking the antigenic epitope-binding groove. Ii protein is later digested in a post-Golgi vesicle and released from the MHC class II molecule in a concerted fashion coupled to the charging of antigenic peptides.[19] One of the major functions of Ii is to protect the antigenic peptide binding site on MHC class II molecules from binding endogenously derived antigenic peptides.[20–22] Suppression of Ii leads to the induction of 'unprotected' MHC class II molecules in an antigen-presenting cell (APC), enabling it to present endogenous antigens by both MHC class I (the normal functional pathway) and 'unprotected' MHC class II molecules, simultaneously activating $CD4^+$ and $CD8^+$ T cells. In an earlier study, we generated a potent sarcoma tumour cell vaccine by inhibiting Ii with Ii anti-sense oligonucleotides.[23] Ii anti-sense oligonucleotides also effectively inhibited Ii expression in dendritic cells (DCs), leading to the presentation of endogenously expressed ovalbumin (OVA) epitopes to $CD4^+$ T cells and a potent tumour vaccine.[24] We have subsequently generated an active Ii suppression plasmid construct: Ii reverse gene construct [Ii-RGC(− 92,97), A in the AUG start codon equals 1], to suppress Ii expression in tumour cells.[25–27] Ii-RGC(− 92,97) codes for expression of an anti-sense mRNA, which hybridizes to the native mRNA for Ii

protein, thereby leading to Ii suppression. This strategy generated MHC class $I^+/II^+/Ii^-$ phenotype tumour cell vaccines in different experimental animal models.[23,25–27]

In this study we have utilized Ii suppression technology to enhance a HIV gp120 DNA vaccine model. Our rationale was that an APC, e.g. DC, that takes up DNA plasmids containing both the gp120 gene and Ii-RGC, will generate $gp120^+$/MHC class II^+/Ii^- DC. 'Unprotected' MHC class II along with MHC class I (the normal functional pathway) molecules will be charged by endogenously produced gp120 epitopes. The DC will subsequently present MHC class II epitopes to activate $CD4^+$ T cells. The enhanced activation of gp120-specific $CD4^+$ T cells will, in turn, help to strengthen the activation of gp120-specific $CD8^+$ T cells, which are sensitized by MHC class I presentation on the same DC, thereby significantly augmenting the efficiency of HIV gp120 DNA vaccines. We report here that addition of the Ii-suppression technology to HIV gp120 DNA vaccine significantly enhances the potency of the gp120 DNA vaccine and serves as a basis for the rational design of human Ii-RNAi constructs, to be used with established DNA vaccines for enhanced antigen-specific $CD4^+$ T cell activation, all of which could potentially have a significant benefit therapeutically or as preventive vaccinations.

Materials and methods

Mice

BALB/c mice (8–12 weeks old) were purchased from Jackson Laboratory and kept in the animal facility at the University of Massachusetts Medical Center, Worcester, MA, USA. All animal procedures were performed following the University of Massachusetts Medical School animal care guidelines under an approved protocol and overseen by the University of Massachusetts IACUC Committee.

Cell lines and antibodies

Murine macrophage J774 cells cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) with 10% fetal calf serum (FCS) were obtained from Dr Gary Ostroff. Anti-murine Ii monoclonal antibody, from culture supernatant, In.1, and anti-murine MHC class II monoclonal antibody, M5/114·15·2, purified from culture supernatant, were used.[28,29]

Plasmids

Murine Ii cDNA[30] was obtained from Dr James Miller of the University of Chicago. Ii-RGC(− 92,97) (numbers represent nucleotide position in Ii cDNA gene, 1 is A in AUG start codon and − 92 represents 5′ upstream 92 nucleotides from AUG) has been described previously.[25–27] The pBudCE4·1 plasmid was purchased from Invitrogen (San Diego, CA, USA). The murine GM-CSF plasmid (pNGVL1-mGM-CSF) was from Dr Gilda G. Hillman of Wayne State University, Detroit, MI and the pCEP4/CI-ITA plasmid[31] was from Dr Laurie Glimcher of the Harvard School of Public Health (Boston, MA, USA). HIV-1 IIIB gp120 cDNA[16] from Dr Norman Letvin at the Beth Israel Deaconess Medical Center (Boston, MA, USA) was cloned into a Rous sarcoma virus (RSV.5) expression vector.[32] Expression of the HIV-1 IIIB gene was confirmed by transfecting RSV.5/gp120 into COS cells (data not shown).

Peptide synthesis

Two peptides, a 15-mer, termed p18 (RIQRGPGRAFVTIGK), and a 10-mer, termed p18–I10 (RGPGRAFVTI), were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA, USA. Peptide p18 is presented by both $H\text{-}2D^d$ and $H\text{-}2A^d$ molecules while p18–I10 is presented only by $H\text{-}2D^d$.[33,34] That is, the shorter p18–I10 peptide contains only the MHC class I-presented epitope while the longer p18 peptide contains both a MHC class II-presented epitope and a MHC class I-presented epitope.

DNA coating of gold particles for gene gun delivery

Plasmid DNA was precipitated onto gold particles. Briefly, 15 mg of 1 μm gold microcarriers (enough for 30 cartridges) (Bio-Rad Laboratories, Inc., Richmond, CA) were resuspended by sonication in 100 μl of 0·1 M spermidine. DNA, at a concentration of 1 mg/ml in endotoxin-free water, was then added and sonicated, after which 200 μl of 2 M $CaCl_2$ was added dropwise. The gold-DNA mixture was allowed to stand for 10 min to precipitate before being washed three times with 1 ml aliquots of 100% ethanol. After the final wash, the pellet was resuspended by vortexing and sonication in 1·86 ml of 100% ethanol. After precipitating, the plasmid DNA was adsorbed onto gold beads and the gold beads were coated evenly onto the inner surface of Tefzel tubing (Bio-Rad). After coating the tubing was then cut into 0·5-inch cartridges. Different DNA loading ratios were designed for respective experiments, as described in Results. Cartridges were stored at 4° with desiccant.

DNA transfection of cells

For gene gun transfection of J774 cells, 10^6 cells in 20 μl of medium were pipetted onto a tissue culture dish in approximately 1 cm diameter circles and then subjected to gene gun shooting with one 0·5-inch cartridge (loaded with 1 μg of DNA) at a helium pressure of 300 pounds per square inch (psi). After culturing at 37° for 42 hr, cells were stained with anti-MHC class II

antibody, M5/114·15·2 or anti-murine Ii monoclonal antibody, In.1, and then with fluorescently labelled secondary antibodies. Stained cells were analysed by fluorescence activated cell sorter (FACS) to determine transfection efficiency.

Gene gun immunization of mice

Prior to vaccinating mice by the gene gun delivery of DNA, each mouse was anaesthetized intraperitoneally (i.p.) with a 50 μl of solution comprising 13 μl ketamine solution (100 mg/ml), 17 μl xylazine solution (20 mg/ml) and 20 μl saline. After anaesthesia, mice were shaved on the abdomen with an electric shaver. The barrel of the gene gun was held directly against the abdominal skin and a single microcarrier shot was delivered using a helium-activated Gene Gun System at 400 psi (PowderJect). Each mouse received three consecutive gene gun inoculations. Two weeks later, mice were boosted with the same amount of DNA by the same method. One week after boost, mice were killed for assays.

Enzyme-linked immunosorbent spot (ELISPOT) assay

Splenocytes were obtained from spleens of the killed mice, according to UMMC IACUC-approved procedures. Immunoaffinity-purified $CD4^+$ and $CD8^+$ splenic lymphocytes (> 95% purity) were obtained from the pooled splenocytes. ELISPOT procedures were the same for all groups. BD Pharmingen kits for murine interferon (IFN)-γ and interleukin (IL)-4 ELISPOT assays were used according to the manufacturer's instructions. Briefly, plates were coated overnight at 4° with the cytokine capture specific antibodies. The plates were then blocked with 10% fetal bovine serum (FBS) in RPMI-1640 for 2 hr at room temperature (RT) and washed four times with 1× phosphate-buffered saline (PBS) containing 0·05% Tween-20 (wash buffer). Freshly isolated single splenocyte suspensions (10^{6}/well) and p18 or p18–I10 peptides (5 μg/well) were added to the anti-cytokine precoated plates. After 42–66 hr of incubation, the plates were washed five times with wash buffer, biotinylated detection antibodies (2 μg/ml) were added and incubated for an additional 2 hr at RT. The plates were washed four times with wash buffer and avidin horseradish peroxidase (avidin-HRP) was added, at a 1 : 100 dilution, followed by a 1-hr incubation at RT. Avidin-HRP was removed by washing four times with wash buffer and two times with 1× PBS. Spots were developed by adding 3-amino-9-ethylcarbazole HRP substrate to the plates for 30 min at RT. Finally, the plates were washed twice with sterile water and dried for 1–2 hr at RT. Digitized images of the spots were analysed with a series 1 Immunospot Analyzer and Immunospot 1·7e software (Cellular Technology Limited, Cleveland, OH, USA).

Statistical analysis

Statistical differences were calculated by Student's *t*-test. Significance was defined as $P < 0{\cdot}05$.

Results

Construction of Ii suppression plasmids

We had previously generated an Ii reverse gene construct Ii-RGC(− 92,97) that effectively inhibited Ii expression in tumour cells and created a potent tumour immunotherapy animal model.[25–27] Ii-RGC(− 92,97) was generated by cloning an Ii gene fragment (− 92,97) (1 is A in AUG) into an expression vector in reverse orientation. The anti-sense RNA produced by this Ii-RGC hybridizes with Ii mRNA to block translation of Ii mRNA and/or triggers the destruction of Ii mRNA.[35,36] In this study, we cloned three copies of the Ii-RGC(− 92,97) gene fragment into one pBudCE4·1 plasmid, generating pBudCE4·1/Ii-RGC(× 3) (Fig. 1) to increase the efficiency of Ii suppression. In pBudCE4·1/Ii-RGC(× 3), each Ii-RGC(− 92,97) gene fragment is driven by a different promoter. The first Ii-RGC(− 92,97) gene fragment was inserted into pBudCE4·1 by *Hin*dIII and *Bam*HI under the control of a cytomegalovirus (CMV) promoter to generate a one-copy pBudCE4·1/Ii-RGC(− 92,97) (pBudCE4·1/Ii-RGC). The second copy of Ii-RGC(− 92,97) was first cloned into the pUB6/V5-His plasmid (Invitrogen, San Diego, CA, USA) under control of a UbC promoter. The UbC promoter, Ii-RGC(− 92,97) fragment and poly A signal sequence were then amplified by polymerase chain reaction (PCR) and cloned into the pBudCE4·1/Ii-RGC to generate a two-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 2)]. The third copy of Ii-RGC(− 92,97) was first cloned into the RSV.5 plasmid under the control of a RSV promoter.[25,27] The RSV promoter, Ii-RGC(− 92,97) fragment and poly A signal were PCR amplified and cloned into pBudCE4·1/Ii-RGC(− 92,97(× 2) to generate a three-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 3)] (Fig. 1). More detailed procedures and enzyme sites for cloning are explained in the legend of Fig. 1.

Ii suppression in macrophage cells by pBudCE4·1/Ii-RGC(× 3)

The activities of the three different pBudCE4·1/Ii-RGC plasmids were tested in COS cells to define the most active pBudCE4·1/Ii-RGC plasmid. This was achieved by determining inhibition of expression of a cotransfected Ii cDNA in COS cells. The results (data not shown) indicated that pBudCE4·1/Ii-RGC(× 3) is most active and has almost completely inhibited Ii expression in transfected COS cells. DCs, macrophages and Langerhans cells play

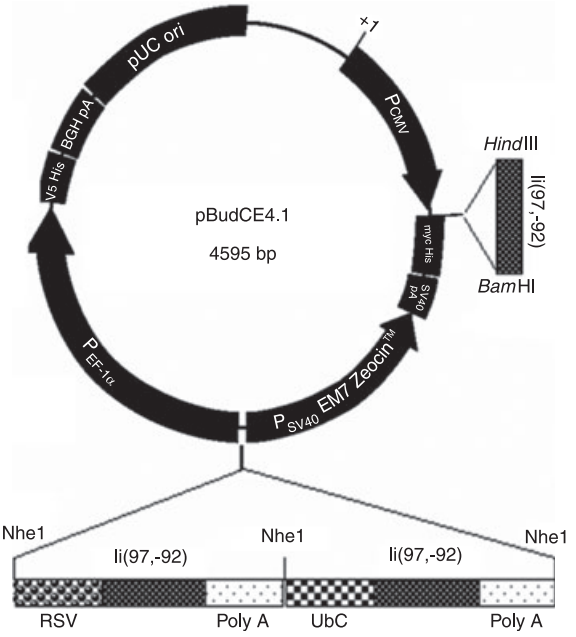

Figure 1. Generation and the map of BudCE4·1/Ii-RGC(× 3). The Ii-RGC(− 92,97) fragment was cloned into the pBudCE4·1 plasmid by *Hin*dIII (97 end, A in AUG codon is 1) and *Bam*HI (− 92 end), under control of a CMV promoter, to create the plasmid one-copy pBudCE4·1/Ii-RGC(− 92,97) (pBudCE4·1/Ii-RGC). For the two-copy Ii-RGC plasmid, the Ii-RGC(− 92,97) fragment was first cloned into pUB6/V5-His by *Hin*dIII (close to the UbC promoter from the 97 end) and *Bam*HI (− 92 end), under control of a UbC promoter, to generate pUB6/V5-His/Ii-RGC(− 92,97). The UbC promoter, Ii-RGC(− 92,97) gene fragment and poly A signal sequence were then amplified by polymerase chain reaction (PCR) and cloned into the Nhe1 site of pBudCE4·1/Ii-RGC to generate a two-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 2)]. For the three-copy Ii-RGC(− 92,97), the Ii-RGC(− 92,97) gene fragment was first cloned into a RSV.5 plasmid by Sal1 (close to the RSV promoter from the 97 end) and *Bam*HI (− 92 end) under control of a Rous sarcoma virus (RSV) promoter to generate RSV.5/Ii-RGC(− 92,97).[25] The RSV promoter, Ii-RGC(− 92,97) gene fragment and poly A signal sequence were amplified by PCR and then cloned into the Nhe 1 site of pBudCE4·1/Ii-RGC(× 2) to generate a three-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 3)]. The figure illustrates that each Ii-RGC(− 92,97) gene fragment was cloned in reverse orientation relative to its promoter. The '97' end of the Ii-RGC(− 92,97) gene fragment is always close to the promoter side, indicating that an anti-sense RNA will be produced. In pBudCE4/Ii-RGC(× 3), each Ii-RGC(− 92,97) gene fragment is driven by a different promoter in order to avoid possible promoter competition.

important roles in the induction of immunity against DNA vaccine antigens, especially when a gene gun is used for DNA delivery.[37,38] For this reason, we tested the activity of our Ii suppression constructs on the murine macrophage line J774. As J774 is an MHC class II-positive and Ii-positive cell line, pBudCD4·1/Ii-RGC(× 3) was used to assess inhibition of endogenously expressed Ii (Fig. 2).

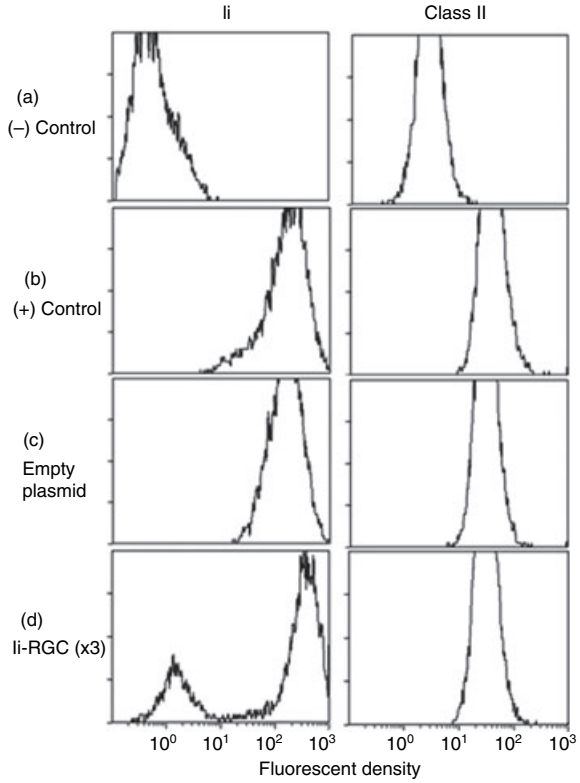

Figure 2. Ii suppression in J774 cells by pBudCD4·1/Ii-RGC(× 3); 10^6 J774 cells were transfected with 1 μg of pBudCE4·1/Ii-RGC(× 3) by gene-gun delivery as described in Materials and Methods. Three replicates of transfected J774 cells (3×10^6) were cultured together for 48 hr, harvested, and stained for both Ii and MHC class II proteins followed by fluorescence activated cell sorter (FACS) analysis. (a) J774 cells stained with only fluorescein isothiocyanate (FITC)-labelled second antibody only (negative control); (b) J774 cells stained with anti-Ii (In.1) or anti-major histocompatibility complex (MHC) class II (M5/114·15·2) antibodies followed by FITC-labelled secondary antibody (positive controls); (c) J774 cells treated with pBudCE4·1 empty plasmid and stained as in (b); (d) J774 cells treated with pBudCE4·1/Ii-RGC(× 3) and stained as in (b). Similar Ii inhibition results were obtained with several other cell lines using gene gun transfection (data not shown).

We normally obtain a 30–70% transient transfection efficiency using the gene gun, depending on the cell line used (unpublished observations). As shown in Fig. 2(d), Ii was significantly suppressed in 31% of transfected J774 cells (> 95% as measured by fluorescence intensity) without apparent change in MHC class II expression by pBudCE4·1/Ii-RGC(× 3). Under normal conditions, the Ii protein is synthesized in excess relative to MHC class II molecules in APC;[39] therefore, > 95% of Ii inhibition could lead to most MHC class II molecules in a transfected DC being unprotected by Ii molecules. These 'unprotected' MHC class II molecules should be available for charging by epitopes (including gp120 epitopes) in the ER, directed to the cell surface, followed by subsequent

presentation to $CD4^+$ T cells. Based on these results pBudCE4·1/Ii-RGC(× 3) was used in all subsequent experiments.

Ii suppression enhances gp120 DNA vaccine efficiency

Next we tested whether Ii suppression enhanced HIV gp120 DNA vaccine efficiency. BALB/c mice were immunized with the gene for gp120, with or without pBudCE4·1/Ii-RGC(× 3). In our *in vivo* experiments, the gene for GM-CSF was included in all groups (except the naive group) as an adjuvant. In Fig. 3, one sees that both p18- and p18–I10-specific ELISPOT assays demonstrated roughly five times the enhancement of IFN-γ secreting cells in the Ii-suppressed groups (groups d and e) compared to the Ii unsuppressed group (group c). Similar reaction patterns to p18 and p18–I10 stimulation were observed, and the p18 peptide gave a greater response in most reactions. This result is consistent with previous reports which show p18–I10 is restricted only by $H\text{-}2D^d$, while the p18 peptide is restricted by both $H\text{-}2D^d$ and $H\text{-}2A^d$.[33,34] The response to p18 reflects both $CD4^+$ and $CD8^+$ reactions and the p18I−10 response reflects only a $CD8^+$ reaction. These results indicate that most IFN-γ spots were produced by $CD8^+$ T cells in ELISPOT with total splenocytes. This is consistent with ELISPOT data using purified $CD4^+$ and $CD8^+$ T cells (next section).

In order to determine whether Ii suppression induced a Th1 or Th2 response, IL-4 secretion was also examined by ELISPOT and was found to be induced in all groups (Fig. 3), and that IL-4 production was enhanced by Ii suppression. As the enhancement pattern of IL-4 is similar to the enhancement of IFN-γ, we conclude that Ii suppression influences the magnitude of the immune response, but does not influence the Th1/Th2 pattern in our model.

Ii suppression enhances the activation of both gp120-specific $CD4^+$ and $CD8^+$ T cells

In order to elucidate clearly whether Ii suppression enhances activation of only gp120-specific Th cells or both Th cells and CTL, we purified $CD4^+$ and $CD8^+$ T cells prior to analysis of cytokine expression. Pooled splenocytes (5×10^6/ml) from each group (five mice) were cultured with p18 peptide (25 μg/ml) for 5 days. The cells were purified with MiniMACs separation units (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for another 48 hr prior to the IFN-γ ELISPOT assay. As indicated in Fig. 4, both $CD4^+$ and $CD8^+$ T cells were activated by gp120 DNA vaccine and Ii suppression enhanced gp120 DNA vaccine activation. This result is consistent with the working hypothesis that Ii suppression enhances the activation of $CD4^+$ Th cells.[40–42] $CD4^+$ T cells in turn augment and strengthen the activation of $CD8^+$ CTL. The frequency of generation of p18-specific $CD4^+$ T cells is consistent with previous reports, showing that the frequency of MHC class II epitope-specific $CD4^+$ Th cells resulting from DNA vaccine is much lower than that of $CD8^+$ T cells.[3,43]

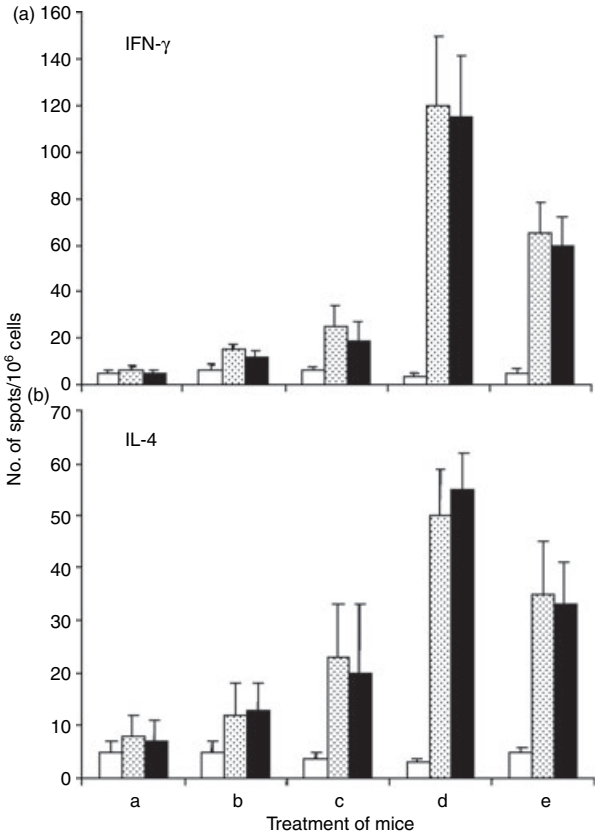

Figure 3. Interferon (IFN)-γ and interleukin (IL)-4 enzyme-linked immunosorbent spot (ELISPOT) assay with splenocytes of mice immunized with gp120 with or without Ii suppression. All groups except (a) (naive mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and each of the following DNA plasmids, respectively: (b) empty pBudCE4·1 (1·35 μg); (c) pNGVL1/GM-CSF (0·35 μg) + empty pBudCE4·1 (1·0 μg); (d) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (0·325 μg) and empty pBudCE4·1 (0·675 μg); (e) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (1·0 μg). Compared to (c), Ii suppression (d) resulted in an approximately five-fold enhancement of IFN-γ secretion. Addition of three times more Ii-RGC(− 92,97)(× 3) (e) did not result in more IFN-γ secretion; instead, IFN-γ secretion in (e) was slightly more reduced than in (d). Splenocytes (10^6/well) from individual mice (five mice/group) were cultured in either medium alone (open bar) or p18 peptide (dotted bar), or p18–I10 peptide (solid bar) in triplicate wells within a precoated IFN-γ ELISPOT plate. Each bar represents the mean ± SD for each of five mice. Immune response enhancement in groups (d) and (e) was statistically significant (compared to group c) ($P < 0{\cdot}05$). Similar results were obtained in three repeated experiments.

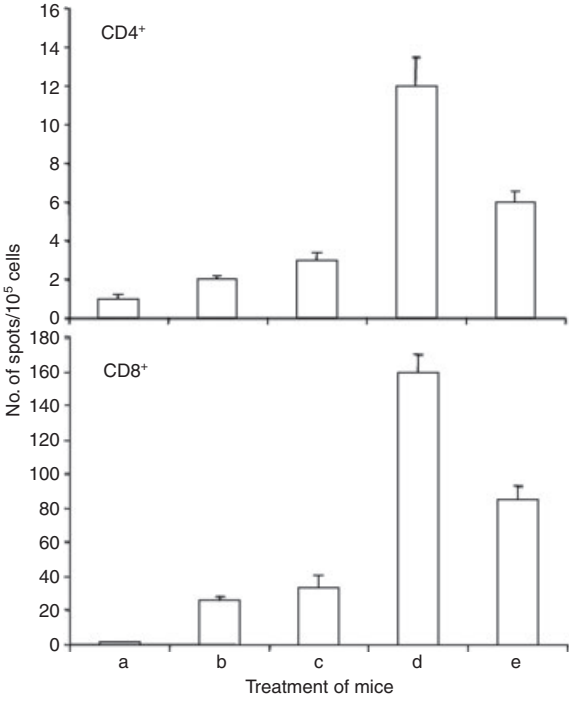

Figure 4. Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assays of immunopurified $CD4^+$ and $CD8^+$ T cells. Splenocytes were pooled together (five mice/group) and incubated (5×10^6/ml) with p18 peptide (25 μg/ml) for 5 days. Then, $CD4^+$ and $CD8^+$ T cells were magnetically isolated using MiniMACS separation units according to the manufacturer's instructions. The purified $CD4^+$ or $CD8^+$ T cells (10^5 cells/well) were cultured along with p18 peptide in triplicate wells within an IFN-γ precoated ELISPOT plate for 48 hr. Each bar illustrates the mean ± SD for triplicate assays. Immune response enhancement in groups (d) and (e) was statistically significant (compared to group c) ($P < 0{\cdot}05$). Group design was the same as for Figure 3. Similar results were obtained in three repeated experiments.

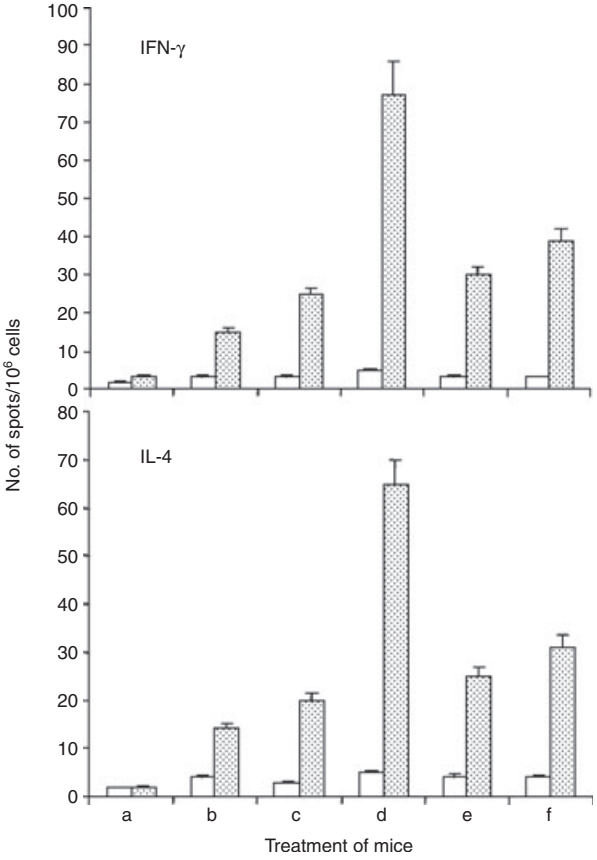

Figure 5. Interferon (IFN)-γ and interleukin (IL)-4 enzyme-linked immunosorbent spot (ELISPOT) assay with vaccine formula in the absence or presence of CIITA. All groups except (a) (naive mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and, respectively, each of the following DNA plasmids: (b) empty plasmid (1 μg); (c) pNGVL1/GM-CSF (0·35 μg) + empty pBudCE4·1 (0·65 μg); (d) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (0·65 μg); (e) pNGVL1/GM-CSF (0·35 μg) + pCEP4/CIITA (50 ng) + empty pBudCE4·1 (0·65 μg); and (f) pNGVL1/GM-CSF (0·35 μg) + pCEP4/CIITA (50 ng) + pBudCE4·1/Ii-RGC(× 3) (0·65 μg). IFN-γ ELISPOT assays were performed with 10^6 cells/well in medium alone (white bar) or stimulated with p18 (dotted bar). Each bar illustrates the mean ± SD for individual mice (five mice/group) tested in groups (c) and (d). Similar results were obtained in three repeated experiments.

Addition of CIITA abolishes the enhancing activity of Ii suppression

Because keratinocytes might play a role in augmenting the magnitude of the immune response to DNA vaccines and are not normally MHC class II-positive,[44–46] the gene encoding MHC class II transactivator (CIITA)[31,47] was added to the DNA used to immunize mice. CIITA is a universal inducer of MHC class II and Ii in a variety of cells[25,47,48] and was used in these experiments to induce MHC class II expression in keratinocytes. Coupling this with Ii suppression increases the frequency of the MHC class II^+/Ii^- phenotype in keratinocytes, which might lead in turn to augmentation of potency of the gp120 DNA vaccine. From Fig. 5, one sees that addition of CIITA (group e) did not enhance the vaccine efficiency (compared to group c); instead, addition of CIITA abolished the vaccine efficiency enhanced by Ii suppression (compare group f with group c). Addition of CIITA also abolished IL-4 enhancement by Ii suppression (Fig. 5). Possible mechanisms for this effect are considered in the Discussion.

Ii suppression enhancement of gp120 DNA vaccine was more profound at the lower concentration of pBudCE4·1/Ii-RGC(× 3) group (Fig. 3, group d). In order to confirm this phenomenon, the medium concentration of pBudCE4·1/Ii-RGC(× 3) plasmid was used (Fig. 5). The three different concentrations of pBudCE4·1/Ii-RGC(× 3) used in experiments of Figs 3 and 5 were compared in Fig. 6 and one can see that the correlation between the doses of pBudCE4·1/Ii-RGC(× 3) and IFN-γ production

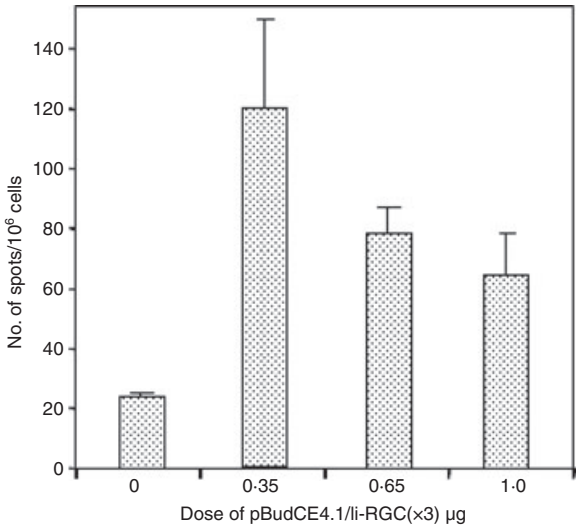

Figure 6. The correlation between concentration of pBudCE4·1/Ii-RGC(× 3) and interferon (IFN)-γ production to P18 stimulation. The IFN-γ production by p18 stimulation data of groups (c), (d) and (e) (dotted bars) in Figure 3 were pooled together with the IFN-γ production data of groups (c) and (d) in Figure 5. The data from the two groups (c) (no Ii-RGC(× 3) plasmid) were identical (25 spots in Figs 3 and 5), indicating good reproducibility of results. One can see more clearly from this figure that the IFN-γ produced by p18 stimulation is well correlated with the dose of pBudCE4·1/Ii-RGC(× 3) used.

indeed exists. The higher the dose of pBudCE4·1/Ii-RGC(× 3) used, the less IFN-γ was produced. This may reflect less promoter competition among gp120-, GM-CSF- and pBudCE4·1/Ii-RGC(× 3).

Discussion

The concept that induction of a MHC class II^+/Ii^- phenotype APC results in the presentation of endogenously derived antigenic epitopes by MHC class II molecules was first developed by Dr Ostrand Rosenberg and colleagues.[40,41,49] They showed that immunization of mice with tumour cells transfected with syngeneic MHC class II molecules (without Ii) led to a potent tumour cell vaccine and that reintroduction of the Ii protein into such tumour cells abolished the induced vaccine potency.[49] Further studies have demonstrated that MHC class II^+/Ii^- tumour cells present cytosolic or ER-retained hem egg lysozyme (HEL) through MHC class II molecules to activate $CD4^+$ T cells and that Ii limits MHC class II presentation.[40] In a recent $CD4^+$ and $CD8^+$ T cell depletion experiment it has further been shown that MHC class II^+/Ii^- tumour cells present endogenously expressed tetanus toxoid to $CD4^+$ T cells but not to $CD8^+$ T cells[42]. Zhao *et al*. have also shown that Ii inhibited DCs present endogenously expressed MHC class II epitopes to $CD4^+$ T cells.[24] Because transfection of tumour cells with a syngeneic MHC class II gene is not clinically feasible, given the great polymorphism of MHC class II genes in humans, we have developed a more practical method based on inhibition of the monomorphic Ii gene. Concomitant administration of Ii-RGC and the gene for CIITA, which is a universal inducer of both MHC class II molecules and the Ii protein,[26,27,47,48] ensures generation of the MHC class II^+/Ii^- phenotype. This method leads to potent tumour cell vaccines when MHC class I is also positive in tumour cells.[23,25–27] In the current study, we applied this strategy to develop a more potent DNA vaccine; i.e. using a construct to suppress Ii in DNA-transfected cells resulting in the presentation of endogenously synthesized vaccine antigens (gp120 epitopes) by 'unprotected' MHC class II, thereby activating $CD4^+$ T helper cells (p18-specific $CD4^+$ T cells). Our results are consistent with previous reports and support the working hypothesis that Ii suppression directly enhances the activation of $CD4^+$ T cells.[27,40,42] Activation of antigen-specific $CD4^+$ T cells is essential for the activation of antigen-specific $CD8^+$ T cells. In this manner, enhanced activation of gp120-specific $CD4^+$ T cells, and in turn enhanced activity of $CD8^+$ cells, is obtained.

Several studies have shown that endogenously expressed antigens can be processed and presented by MHC class II molecules.[50–52] Lepage *et al.*[52] have demonstrated that endogenous expressed membrane gp100 can be presented by both MHC class I and class II molecules in the absence of Ii. Deletion of the signal and transmembrane sequences decreased the class II presentation without affecting class I presentation. This result indicates that membrane antigens can be presented by MHC class II, as is the membrane protein gp120. Secondly, studies by us and others have indicated that MHC class II^+/Ii^- phenotype tumour cells are potent tumour cell vaccines,[23,25,27,40,41] while MHC class II^-/Ii^- and MHC class II^+/Ii^+ tumour cells are not, indicating that Ii limits the presentation of endogenous tumour antigens. Armstrong *et al.* have shown that Ii inhibits the ER-retained HEL epitope to be presented by MHC class II to $CD4^+$ T cells.[40] Zhao *et al*. have shown that Ii inhibition enhances DC to present endogenously expressed OVA epitopes through MHC class II and leads to enhanced activation of $CD4^+$ T cells, and subsequently to an enhanced $CD8^+$ CTL activity.[24] However, Thompson *et al.* found that Her2/neu MHC class II epitopes are not limited by Ii.[48] These discrepancies can be well explained by the direct chemical evidence of Muntasell *et al.*[53] Their mass spectroscopy study has revealed that MHC class II molecules of II^+ Ii^- cells contain epitopes presented by MHC class II^+ Ii^+ cells plus additional novel peptides which are not presented by MHC class II^+ Ii^+ cells. This direct evidence indicates that some endogenous epitopes are limited by Ii and some are not. Our experimental results indicate that p18 is limited by Ii.

Ii suppression enhancement of gp120 DNA vaccine seems to correlate with the dose of pBudCE4·1/Ii-RGC(× 3) plasmid used (Fig. 3). In order to define whether the dose of pBudCE4·1/Ii-RGC(× 3) is correlated with the enhancement of gp120 DNA vaccine efficiency, we used a middle dose of pBudCE4·1/Ii-RGC(× 3) in a further experiment (Fig. 5). Combining the data of Fig. 3 (groups c, d and e) with the data from Fig. 5 (groups c and d), we have drawn Fig. 6. One sees that the dose of pBudCE4·1/Ii-RGC(× 3) is indeed correlated with enhancement of gp120 DNA vaccine efficiency. The mechanisms underlying this phenomenon are unclear. Promoter competition could be a potential explanation.[54] The construct pBudCE4·1/Ii-RGC(× 3) contains an RSV promoter that shares with the RSV/gp120 plasmid.

Gene gun-mediated DNA immunization results in the transfection of keratinocytes and local DC.[5,38] Porgador *et al.*[55] demonstrated that after gene gun delivery of DNA, 20 000–30 000 DCs were recruited per draining lymph node and that 20–75 DCs were directly transfected with the administered DNA. Furthermore, they showed that the transfected DCs are the predominant APCs for CTL activation. Akbari *et al.* showed that DNA vaccination led to a relatively low frequency of DC transfection.[56] However, it was these transfected DCs that led to the general activation of all DCs, providing good conditions for effective Th cell activation. Our hypothesis is that gene gun-mediated vaccination of mice with pBudCE4·1/Ii-RGC(× 3) plus pcDNA(3)/gp120 led to the generation of gp120$^+$/MHC class II$^+$/Ii$^-$ DCs, that are more effective in activating gp120-specific CD4$^+$ Th cells through the presentation of endogenously synthesized gp120 epitopes by 'unprotected' MHC class II molecules.

Other studies have indicated that local cells (e.g. keratinocytes) also play a key role in the induction of the humoral and CTL activities of a DNA vaccine. Ablation of the injected skin or local inhibition of gene expression abolished the efficacy of a DNA vaccine.[44–46] We added the CIITA gene to the Ii-RGC/gp120 DNA vaccine in the hope of increasing the frequency of MHC class II$^+$/Ii$^-$ keratinocytes, further enhancing the DNA vaccine potency. However, we found that the addition of the CIITA gene did not improve efficacy of the vaccine (compare group c to group e in Fig. 5) and, instead, decreased the vaccine potency enhancement imparted by Ii suppression (compare group d to group f in Fig. 5). Our previous experiments have shown that three times more Ii-RGC plasmid is needed to suppress Ii induced by CIITA (unpublished observation). We conclude that the MHC class II$^+$/Ii$^-$ phenotype of keratinocytes in our *in vivo* experiment is induced by our vaccine as the concentration of CIITA plasmid was 13 times lower than the Ii-RGC plasmid (see Fig. 5 legend). Mechanisms to explain this phenomenon relate to the findings of Landmann *et al.*[57] They demonstrated that in the process of DC maturation, there is enhanced cell surface MHC class II expression followed by the *de novo* biosynthesis of MHC class II mRNA being turned off. This is due to a rapid reduction in the synthesis of CIITA, triggered by a variety of different maturation stimuli, including lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α, CD40 ligand, IFN-α and infection with *Salmonella typhimurium* or Sendai virus.[57] The addition of CIITA could potentially block the *de novo* process that turns off MHC class II mRNA, disturbing the normal maturation of DCs. Our data indicate that keratinocytes are not major APCs for a DNA vaccine under our condition, because increased antigen presentation by MHC class II$^+$/Ii$^-$ keratinocytes did not enhance the efficiency of gp120 DNA vaccine.

The major advantage of using Ii suppression to augment MHC class II presentation of endogenously expressed DNA antigens is that it induces strong antigen-specific CD4$^+$ T cell activation, while at the same time the induction of CD8$^+$ T cells continues uninterrupted. Vaccine antigens are also released and phagocytosized by DCs or other APCs, through the exogenous antigen processing and presentation pathway to activate Th cells.[58] However, the availability of soluble antigen to MHC class II molecules is limited by the low levels of released antigen; while these antigens need to be taken up by APCs and DCs throughout the body, losing the advantage of colocalized stimulation of CD4$^+$ and CD8$^+$ cells. Our Ii inhibition strategy leads to a simultaneous transfection of DCs with DNA containing HIV gp120 and Ii-RGC, resulting in the expression of gp120 and 'unprotected' MHC class II molecules in a single DC. Following endogenous synthesis of gp120, processing and presentation of gp120 epitopes through MHC class I and class II molecules occurs simultaneously. This results in a stronger localized CD4$^+$ and CD8$^+$ T cell collaboration to increase efficacy of the DNA vaccine.

Our results support the feasibility of a novel strategy to augment the efficacy of DNA vaccines in a clinical setting. We have generated active human Ii-RNAi constructs for just such a purpose. Ii suppression technology does not conflict with other vaccine enhancement technologies and can be used in combination with other vaccine methods, including cytokines and adjuvants to further enhance DNA vaccines.

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